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Morphological, Ultramorphological and Molecular Preliminary Evaluation of Midwest Portuguese Populations of *Rhipicephalus sanguineus sensu lato*

Maria João Coimbra Dores

Dissertação

Mestrado em Biologia Humana e Ambiente

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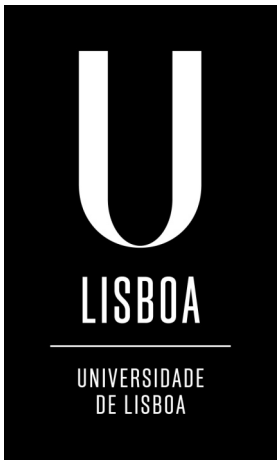

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Orientadores: **Doutora Fernanda Rosa**, Instituto de Investigação Cientifica Tropical, Rua da Junqueira, nº14, 1300-343 Lisboa, Portugal

Professora Doutora Deodália Dias, Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, 1749-016 Lisboa, Portugal

2014

Aos meus pares de pais e aos meus avós, meus grandes professores da arte da vida. Ao Quincas, à Ana e ao Gonçalo, aqueles que sempre caminham ao meu lado.

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Os Agradecimentos e o Sumário não seguem as novas regras do acordo ortográfico.

As referências bibliográficas nesta dissertação estão de acordo com as normas da revista *Journal Tick and Tick-borne Diseases*.

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe o quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive."

Ricardo Reis

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e me inspirou a ter uma dedicação feroz e uma persistência inabalável entre muitas outras coisas.

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SUMÁRIO

As carraças (Ordem Ixodida: Classe Arachnida) são artrópodes hematófagos estritos e ectoparasitas de uma grande variedade de vertebrados terrestres com uma grande importância médica e veterinária pois, para além de poderem causar lesões aos seus hospedeiros devido à sua acção hematófaga, podem também transmitir vários agentes patogénicos aos seus hospedeiros. Encontram-se vasta e mundialmente distribuídos, apesar de se concentrarem especialmente nas regiões mais temperadas do planeta.

Embora sejam caracterizados como vectores de zoonoses, são várias as espécies de carraças associadas à transmissão de agentes patogénicos ao Homem responsáveis pelo aparecimento de diversas doenças infecciosas como rickettsioses, ehrlichioses, febre botonosa, leishmaniose, entre outras patologias.

A determinação e diferenciação de espécies de ixodídeos são tradicionalmente baseadas em características morfológicas, ecológicas e biológicas dos espécimes em estudo. Infelizmente, estas metodologias são substancialmente limitadas, não só pela presença de variações inter e intraespecíficas, como pela existência de grandes semelhanças ecológicas, biológicas e geográficas, dificultando assim o diagnóstico.

Com o desenvolvimento de técnicas de biologia molecular, como a reacção de polimerase em cadeia (RPC) a análise de marcadores específicos no ácido desoxirribonucleico (ADN) dos ixodídeos permite detectar e evidenciar a variabilidade inter e intraespecífica e, assim, obter informação que permita distinguir os diferentes polimorfismos que caracterizam as diferentes espécies de carraças.

Dentro das três famílias de ixodídeos existentes no mundo, é a família Ixodidae (carraças de corpo duro) a que tem maior importância médica/veterinária, devido ao grande número de espécies nela incluída ligada à transmissão de agentes patogénicos. Esta inclui o género *Rhipicephalus* (Subfamília Rhipicephalinae), um dos considerados mais controversos em termos taxonómicos e, simultaneamente, dos mais distribuídos mundialmente. A espécie *Rhipicephalus sanguineus* (originalmente descrita por Latreille em 1806) é uma das 84 espécies do género, e apresenta uma grande diversidade morfológica intraespecífica, a qual tem sido assinalada por vários autores. Ela encontra-se incluída e é o holótipo do designado grupo ou complexo *R. sanguineus* (*R. sanguineus sensu lato*), que inclui pelo menos 11 espécies (3 delas assinaladas em Portugal) que apresentam diversas parecenças

biológicas e morfológicas. Entre elas está incluída a espécie *R. turanicus*, a qual é considerada a mais similar à espécie *R. sanguineus sensu stricto*, sendo inclusivamente consideradas como uma única espécie presente em Portugal por alguns autores. O diagnóstico preciso é importante nestes artrópodes, já que diferentes espécies apresentam diferentes capacidades vectoriais na transmissão de agentes patogénicos, e suspeita-se que a espécie *R. sanguineus* seja o que tem maior interação com o homem.

Em Portugal, esta espécie já foi detectada de norte a sul do país, e é responsável pela transmissão de diversos agentes patogénicos, entre os quais *Babesia canis* (causa babesiose canina), *Ehrlichia canis* (causa ehrlichiose canina) e *Ricketsia conorii* (causa de febre escaro-nodular ou botonosa no homem). É então de importância médica e veterinária aumentar o conhecimento científico relativamente a aspectos de caracterização morfológica, taxonómica e genética das populações portuguesas destas carraças como vectores, de forma a melhor compreender os aspectos biológicos, ecológicos e epidemiológicos destas carraças no país, sendo um tema com interesse económico e de saúde pública.

Assim, realizou-se um estudo preliminar morfológico e filogenético da espécie *R. sanguineus sensu lato* colectadas em cães provenientes de três concelhos portugueses: Óbidos, Caldas da Rainha e Santarém. Este estudo foi feito em quatro fases: a análise morfológica via microscopia estereoscópica baseada na avaliação tradicional dos espécimes e na classificação mais recente da variedade do grupo *R. sanguineus*, a análise estatística de caracteres morfológicos considerados dos mais discriminatórios com vista na formação de grupos de semelhança (Análise Hierárquica de Clusters), análise ultramorfológica por microscopia electrónica de varrimento (MEV) baseada na classificação taxonómica mais recente da variedade do grupo *R. sanguineus*, e pela análise molecular baseada no marcador de ADN mitocondrial citocromo c oxidase I (COI).

Os resultados obtidos com todas as metodologias mencionadas excepto a análise molecular que não foi conclusiva, e após comparação, permitem-nos concluir e confirmar que existe uma grande variedade morfológica relativa à espécie *R. sanguineus s.l.* nas populações portuguesas estudadas, sendo que a presença da espécie *R. turanicus* em Portugal é fundamentada, e é sugerida assim uma nova classificação segundo semelhanças taxonómicas observadas no grupo.

Assim sendo, nos machos obtiveram-se 8 grupos morfologicamente distintos, dos quais 4 não descritos na mais recente classificação morfológica para *R. sanguineus*. Por outro lado, obtivemos 7 grupos morfológicos para as fêmeas, não tão distintos quanto os dos machos, dos quais 4 não descritos também na mais recente classificação morfológica para *R. sanguineus*.

É também sugerido o uso futuro da metodologia estatística empregue como técnica de avaliação da variabilidade morfológica de uma população, já que esta se provou um auxiliar poderoso no que toca a avaliação da variabilidade presente dentro de uma população em estudo.

Palavras-chave: *Rhipicephalus sanguineus*, Caracterização morfológica, Variabilidade intraespecífica, Análise molecular, Populações Portuguesas.

ABSTRACT

Rhipicephalus sanguineus, commonly known as “brown dog tick” or “kennel tick”, is a three-host tick that parasitizes mainly dogs and occasionally humans. This arthropod is worldwide distributed and is a vector of some zoonosis, as *Rickettsia conorii* (pathogen of Mediterranean spotted fever).

In Portugal, *R. turanicus* present high morphological resemblances to *R. sanguineus*, and as both populations are sympatric and very genetically similar, are easily misidentified. However, through a careful morphological analysis, it is possible to distinguish them, especially by the use of electronic microscopy.

Although some prior molecular and morphological studies have already been performed on these Portuguese populations, the results differ, which means there is still much speculation and disagreement around this taxonomic classification.

In this context, this study was conducted using morphological (stereoscopic microscopy), ultramorfological (scanning electron microscopy - SEM) and molecular analysis (COI was the molecular marker of choice) in order to clarify the taxonomic status of these populations. For that purpose, three *R. sanguineus s.l.* populations collected from the districts of Óbidos, Santarém and Caldas da Rainha were used.

The species identification was performed based on morphology characteristics, using standard taxonomic keys (Dias, 1994; Papadopoulos et al., 1992; Walker et al., 2003). Ticks that could not be morphological identified as *R. sanguineus* or *R. turanicus* were defined as intermediate species. Some *R. pusillus* specimens were included in the analysis as an out-group.

The results obtained with the mentioned methods (except for the molecular analysis), allowed to conclude that there is a great morphological diversity within *R. sanguineus s.l.* Portuguese populations, and that the presence of *R. turanicus* in Portugal is grounded. Thus, it is suggested a new taxonomic classification based in observed variability within the group. It also suggested the future use of the statistical methodology employed as a technique for morphological variability evaluation within a representative population.

Keywords:, *Rhipicephalus sanguineus*, morphological characterization, Intraspecific Variability, Molecular analysis, Portuguese Populations.

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LIST OF ABBREVIATIONS

ADN -	Ácido desoxirribonucleico
CA -	Correspondence Analysis
COXI -	Cytochrome c oxidase I (alternative abbreviation)
COI -	Cytochrome c oxidase I (English) or citocromo c oxidase I (Portuguese)
COI-F -	Cytochrome c oxidase I foward
COI-R -	Cytochrome c oxidase I reverse
DNA –	Deoxyribonucleic acid
EF1α –	Elongation factor 1 alpha
HCA -	Hierarchical Cluster Analysis
IICT -	Instituto de Investigação Cientifica Tropical
IOTU -	Integrated operational taxonomic units
ITS –	Internal transcribed spacer
ITS1 –	Internal transcribed spacer 1
ITS2 –	Internal transcribed spacer 2
LAS –	Leica application system
LS -	Light microscopy
MSF –	Mediterranean spotted fever
MEV –	Microscopia electrónica de varrimento
mtDNA –	Mitochondrial deoxyribonucleic acid
NCR –	Non-coding region
Numt –	Nuclear mitochondrial deoxyribonucleic acid
PA –	Pathogenic agents
PCR –	Polymerase chain reaction
RPC –	Reacção de polimerase em cadeia
R. sanguineus -	*Rhipicephalus sanguineus* (being *R*. the abreeviation to *Rhipicephalus*, and is the same thing for all the othe scientific tick names)
R. sanguineus s.l . -	*Rhipicephalus sanguineus sensu lato*
R. sanguineus s.s. -	*Rhipicephalus sanguineus sensu stricto*
RNA –	Ribonucleic acid
rDNA –	Ribosomal deoxyribonucleic acid
rRNA –	Ribosomal ribonucleic acid
SEM –	Scanning electron microscopy
SPD –	Spotted fever disease
SFGR –	Spotted fever group rickettsias
TBD –	Tick-borne diseases
tRNA -	Transfer ribonucleic acid
USA –	United States of America
USA –	United States of America
WHO –	World Health Organization
ZC -	Zoological Collection

1. INTRODUCTION

The ticks have awaken the scientific attention worldwide since the acknowledgement that they can transmit pathogens to both animals and humans. In recent years, the research conducted on interactions between tick-vectors, hosts and pathogens has been greatly enhanced, all due to the public health problems and economic losses associated with tick-borne diseases.

Besides being spread worldwide, this arthropods can transmit a greater variety of pathogenic agents (PA) to their hosts (such as fungi, viruses, bacteria, protozoa, and helminthes) (Arthur, 1962; Dantas-Torres et al., 2012; Pathak, 1987; Santos-Silva et al., 2013; Shaw et al., 2001; Sonenshine and Roe, 2014) than any other arthropod group (Pathak, 1987; Ribeiro et al., 1996; Santos-Silva et al., 2013; Sonenshine and Roe, 2014), and can parasitize members of every single class of terrestrial vertebrates including birds, reptiles, amphibians, and mammals (humans included) (Carpenter et al., 1990; Pathak, 1987; Santos-Silva et al., 2013; Sonenshine and Roe, 2014; Szabó et al., 2008). Moreover, the incidence of canine and human tick-borne diseases (TBD) have been increasing in Europe in the past few years (Földvári, 2005; Santos-Silva et al., 2013), and the issues related with the correct identification of some vectors involved are still unclear.

Ticks are also an important cause of economic losses, being their infestation injurious to livestock animals once it can lead to reduced weight gain, loss of milk production or even abortion, and in some regions of the world this is such a threat that livestock production is almost impossible without major investments in tick control (Marcelino et al., 2012; Sonenshine and Roe, 2014).

Amongst tick groups, the genus *Rhipicephalus* Koch (1844) (Ixodida: Rhipicephalinae) is one of the most important, however its members are difficult to characterize and it concurs with a controversial taxonomic characterization (Pegram et al., 1987b; Walker et al., 2000). This makes even more evident the need of clarifying this matter. This is the case, in particular, of the *Rhipicephalus sanguineus* group that has been for a long time a questionable subject of research (Gray et al., 2013; Pegram et al., 1987a, 1987b; Santos-Silva et al., 2011). The main issue regarding this group is that the original-specimen of *R. sanguineus* – *R. sanguineus sensu stricto* (*s.s.*) – has been lost and the description made by Latreille (1806) is no longer suitable: “blood red, punctate, posteriorly with three

impressed lines; no distinct thoracic spot antero-dorsally". Therefore, a reliable taxonomic definition of this species is currently lacking. The confusion reported in the identification of some *Rhipicephalus* spp. throughout the years (Barker, 1998; Estrada-Peña and Sánchez, 1988; Murrell and Barker, 2003; Pegram et al., 1987b; Rosa et al., 2013; Santos-Silva, 2010) can easily jeopardize the studies to be on this topic due to insufficient or unreliable data collected.

In Portugal, at least 21 ticks' species exists, among which the *R. sanguineus* is the most widespread and the more suitable to convey a greater variety of PA (Santos-Silva et al., 2013). Nevertheless, it seems that a considerable amount of ticks currently identified as *R. sanguineus* (Santos-Silva, 2010) might actually represent other closely related species (for example *R. turanicus*) (Dantas-Torres, 2010; Pegram et al., 1987a, 1987b; Rosa et al., 2013), being this confusion due to its morphological similarities (as happens with *R. camicasi*), although theoretically they present different biological behaviours, ecological characteristics and vector capacities (Estrada-Peña et al., 2004; Walker et al., 2000).

Therefore, one could state that the main current taxonomic issue is that the described morphological variation within *R. sanguineus* (which ultimately can include *R. turanicus*) cannot be accommodated in the currently acknowledged variability of these species (Gray et al., 2013; Rosa et al., 2013). Thus it means that the *R. sanguineus* present in Portugal have to be classified as *R. sanguineus sensu lato* (*s.l.*), once no agreement on this *R. sanguineus* populations have been achieved yet. Complicating this matter further, its deoxyribonucleic acid (DNA) features are more similar to those reported for other species, and some authors claim that some sequences available in GenBank may be inaccurate due to incorrect morphological identification (Dantas-Torres et al., 2013; Gray et al., 2013).

Amongst the species with economic and public health importance, the right acknowledgment of *R. sanguineus s.l.* morphological variations and its correct taxonomic identification is paramount from an epidemiologic point of view, because even distinct populations of the same species can have a variable parasitic specificity and variable vector capacity (Dantas-Torres et al., 2012; Walker et al., 2000). That reinforces the importance of clarifying the Portuguese *Rhipicephalus*' biosystematics. In this context, and taking in consideration the diversity of ecological distinct geographical regions, this investigation on Portuguese population of *R. sanguineus s.l.* comes as a preliminary work

aimed to thoroughly assess possible meaningful morphological variations that suggest the existence of distinct populations or even distinct species of the *R. sanguineus* group.

1.1. Biology and Ecology of *R. sanguineus*: Overview

Ticks are one of the most familiar dog-parasites, especially to those of the tropical and temperate regions of the world (Dantas-Torres, 2008; Estrada-Peña and Santos-Silva, 2005; Goddard, 1987; Jacobs et al., 2001; Labuda and Nuttall, 2004; Louly et al., 2006, 2007; Walker et al., 2000).

R. sanguineus is commonly known as the "brown dog tick" because it parasitizes mainly dogs (it is a monotropic tick – all development stages can feed on the same host species), although it can be often found on cattle, horses, goats, cats, wild animals, and on humans (Dantas-Torres, 2010; Pegram et al., 1987a; Szabó et al., 2005; Walker et al., 2000). Ticks collected from domestic and wild animals might eventually resemble *R. sanguineus* but actually represent other species, such as *R. turanicus*, being this last one often found on cattle, horses, goats, cats, and a range of wildlife species (Walker et al., 2000).

R. sanguineus usually reproduces itself up to 2 generations in a year period, but under favourable conditions they can even complete up to 3 or 4 generations each year, what is the case in Brazil (Labuda and Nuttall, 2004; Louly et al., 2007; Silveira et al., 2009). This tick can also remain active over winter in tropical and sub-tropical countries (such as in Brazil and in southern Europe), and due to their exceptional longevity it can carry pathogens over longer periods of time, what implies that these species are a vector and an excellent reservoir-host for the PA they carry (Labuda and Nuttall, 2004).

The increasing circulation of pets have created the opportunity for *R. sanguineus* species to explore new niches in different climatic and geographic conditions, giving them access to new natural environments in which can proliferate on and, additionally, the increment of hosts-species populations associated to human activity (such as domestic animals and livestock) have all eventually resulted in the increase of this ticks' population and in its fast spread distribution worldwide (Gray et al., 2013; Shaw et al., 2001).

Despite being most common in the temperate parts of the world, this tick species can survive in buildings with heat control systems present in urban communities in the

northern hemisphere of the globe, being well adapted to live in human dwellings. They were probably introduced in this regions specially through imported or travelling dogs come from endemic areas (Claerebout et al., 2013; Dantas-Torres, 2008; Russell et al., 2013; Szabó et al., 2008).

R. sanguineus is so an endophilic tick, being a parasite mostly found in indoors environments, nevertheless it can also colonize peridomestic areas (as parks and kennels) (Dantas-torres et al., 2006; Demma et al., 2006; Nicholson et al., 2006) if the weather is suitable and there is availability of hosts.

In conclusion, the *R. sanguineus* vast worldwide distribution is a consequence of its ability to adopt different survival strategies (Dantas-Torres, 2010), which was made possible by its capacity of regulating the life cycle accordingly to the provided environmental conditions.

1.1.1. Development Stages/ Life Cycle

Ixodid ticks have a long biological cycle that can last from 3-6 months up to 3 years, and comprises four developmental stages: egg (the only inactive phase), larva, nymph and adult, that are represented in Fig.1 (Arthur, 1962; Santos-Silva et al., 2013; Sonenshine and Roe, 2014).

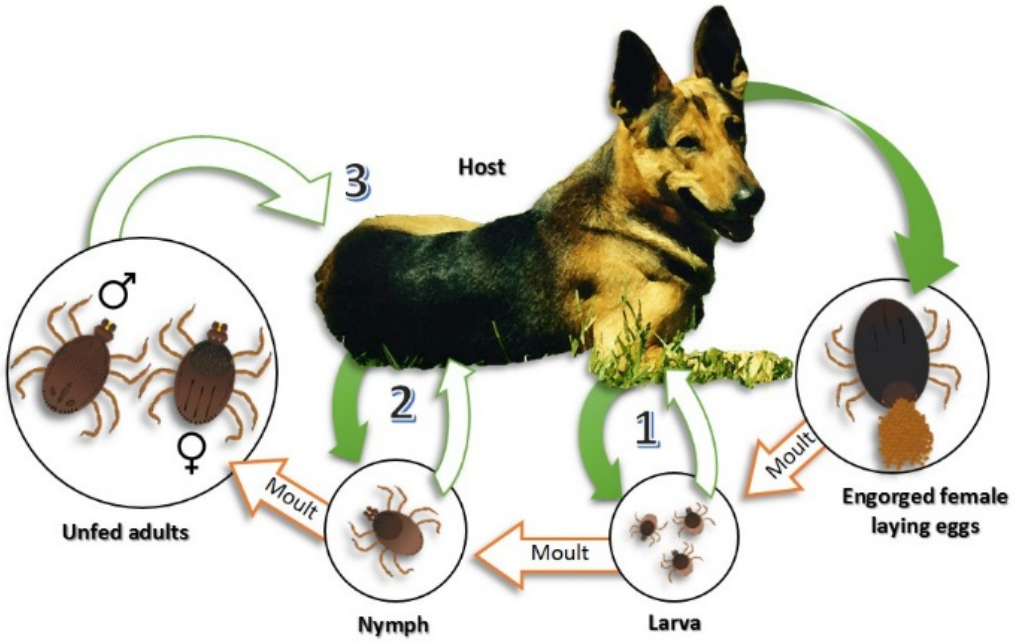

Fig. 1 – Three-host life cycle of *R. sanguineus*. The moult or ecdysis takes place after a host-blood meal and between life stages (larva-nymph and nymph-adult). 1, 2 and 3 represent the host-attachments needed between moulting phases, necessary to engorge.

Ticks also exhibit hemimetabolous development, that is an incomplete metamorphosis (also called moult or ecdysis) in which the body of immature individuals pass by two phases that eventually lead to the adult form by shedding out an outer cuticular layer (Dantas-Torres, 2010; Sonenshine and Roe, 2014). The variations between the different forms are the number of legs that pass from 6 in the larva to 8 in the nymph and adult stages; the sexual development, where sexual dimorphism is reached in adulthood (see Fig.2 and Fig.3Fig.3); the hard chitinous shield (called scutum) that is located in the anterior dorsal surface of larvae, nymphs and females, while in adult males this shield (called conscutum) occupies the entire dorsal surface (see Fig.2); and finally the porose areas appearance on adult females basis capituli (see Fig.2) (Dantas-Torres, 2010; Pathak, 1987; Santos-Silva et al., 2013; Walker et al., 2003).

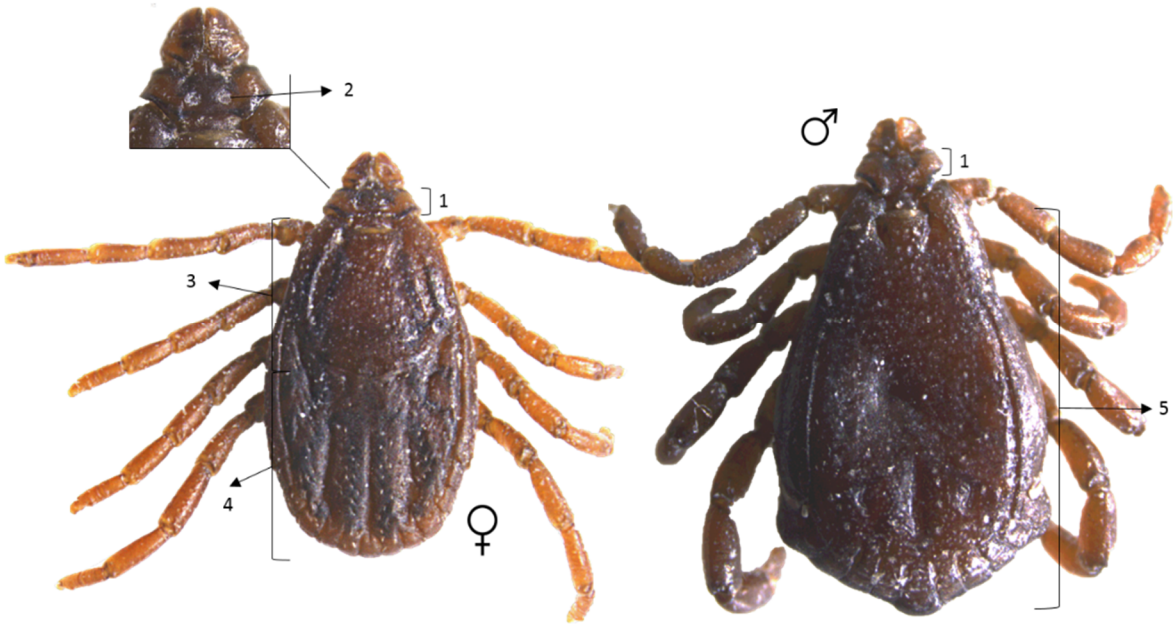

Fig.2 – Presence of sexual dimorphism between *R. sanguineus* genders: dorsal view. Note the presence of four pairs of legs in both adult specimens. (1) Basis capituli, (2) Porose areas, (3) Scutum, (4) Alloscutum, (5) Conscutum (scutum united with alloscutum in the *R. sanguineus* adult males).

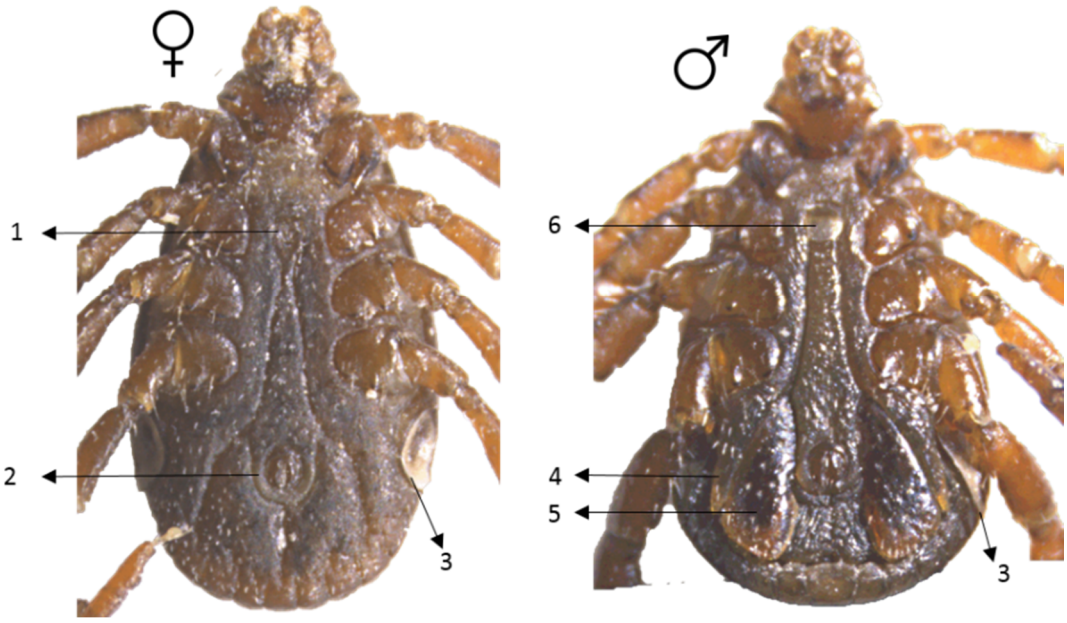

Fig.3 – Presence of sexual dimorphism between *R. sanguineus* genders: ventral view. Note the differences between both adult specimens depicted in the anus and genital areas. (1) Genital aperture, (2) Anus (in the same area in both genders), (3) Spiracular area (it differs in form between the two genders), (4) Accessory adanal plates, (5) Adanal plates, (6) Spermatheca growing area.

The moulting process can take several intervals of time, depending on factors such as life stage (the interval is greater in nymphs than in larvae) and weather conditions (once a stressful temperature and humidity environment can extend the moulting period – low temperatures, for example 10ºC, may lead immature stages to diapause, while higher temperatures accelerate the moulting period) (Inokuma et al., 1997; Koch and Tuck, 1986). This process is regulated by moulting hormones, called ecdysteroids (Rees, 2004), and takes place between life stages (larvae-nymph and nymph-adult), enabling the developing tick to expand within a new external skeleton (Dantas-Torres, 2010; Pathak, 1987), as can be seen in Fig.4a.

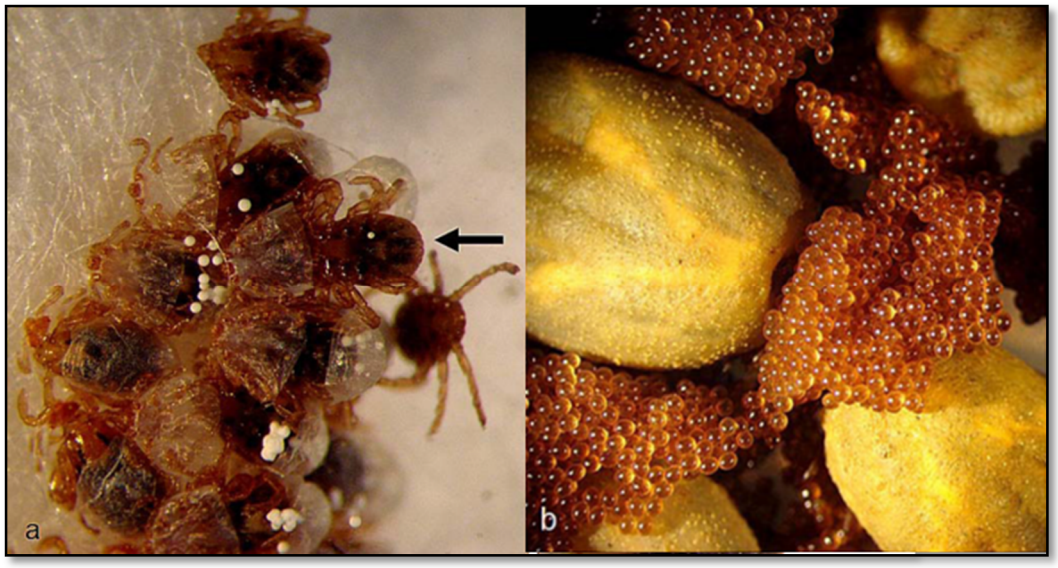

Fig.4 – Moulting and oviposition of *R. sanguineus*, adapted from Dantas-Torres (2010). (a) Larva moulting to nymph (arrow), (b) Several engorged females laying eggs under laboratory conditions (26ºC, 80% humidity).

After the ecdysis, adult ticks seek for hosts, attach, feed, mate and, in the case of females engorgement, drop off to lay their eggs (between 1500 and 5000 eggs in the case of *R. sanguineus*) and eventually die (which is called a single gonotrophic cycle) (see Fig.4b) (Dantas-Torres, 2010; Santos-Silva et al., 2013; Sonenshine and Roe, 2014). Every male tick specimen will attempt to mate with as many females as it can, taking several small meals between the transference of the sack of sperm – spermatheca – to the female (Little et al., 2007; Walker et al., 2003).

Every time *R. sanguineus* ticks reaches sexual maturity and mates, they are lodged on a host (Dantas-Torres, 2010). Mated ixodid females are the only ones that can achieve the full engorged state (see Fig.4b), and may engorge to approximately 100 times their original body weight before dropping off in a sheltered place, provided with enough sperm stored to fertilize their eggs (Dantas-Torres, 2010; Sonenshine and Roe, 2014; Walker et al., 2003). Mating coincides with periods of hosts availability and favourable environmental conditions to the rapid tick population growth (Dantas-Torres, 2010; Figueiredo, 2008; Sonenshine and Roe, 2014).

It means that, during the active phases, ticks alternate between periods of intense activity (questing for hosts, mating and feeding) and non-active periods (when they moult or diapause), requiring blood meals to moult, lay eggs, and mate (Dantas-Torres, 2010; Pathak, 1987; Santos-Silva et al., 2013, 2006).

To obtain blood, ticks cut the host's skin with their chelicerae, insert its hypostome (physiognomic toothed part) to anchor themselves to the host skin, remaining attached by a secreted cement substance that glues them to the surrounding host skin and by their mouthparts for extended periods (minutes, hours or even days), while the feeding process is occurring (feeding period of *R. sanguineus* can last between 2 to 14 days, depending on tick developmental stage and host-species) (Little et al., 2007; Sonenshine and Roe, 2014; Walker et al., 2000). The host's reaction to this physical and chemical assault comprises haemostatic, inflammatory and immune responses (Labuda and Nuttall, 2004).

The success of this arthropod feeding method is based on its complex salivary glands and secreted saliva, where the main PA are found (Labuda and Nuttall, 2004).

1.1.2. Host Specificity

As referred previously, *R. sanguineus* parasitizes mainly dogs, both in urban and rural farming areas (Dantas-Torres et al., 2004; Szabó et al., 2001). Being their natural host, the presence of dogs is a necessary condition in the maintenance of *R. sanguineus* population and, as human pets, an important reservoir of human TBD (Labuda and Nuttall, 2004; Marcelino et al., 2012; Pegram et al., 1987b; Rosa et al., 2006; Shaw et al., 2001).

It is difficult to domestic animals to gain resistance to *R. sanguineus*, probably because of the *R. sanguineus* ticks-saliva chemicals (such as salivary anti-histaminic activity) that enables the decreasing of natural host immune capacity (Bechara et al., 1994; Dantas-Torres, 2008; Inokuma et al., 1997; Jittapalapong et al., 2000; Labuda and Nuttall, 2004). Moreover, some dog breeds apparently seem to be more prone to achieve immune resistance than others (Louly et al., 2010). This could mean that some domestic dog breeds are more host-specific to *R. sanguineus* than others.

Ticks find their hosts by detecting animals' breath and body odors, or by sensing body heat, moisture and vibrations, using their developed sensorial system (such as Haller's organ) (Arthur, 1962; Dantas-Torres, 2010). Once the target is choosen, the tick waits on the tips of grasses and shrubs, holding onto leaves and grass by their third and fourth pair of legs (Arthur, 1962; Dantas-Torres, 2010). When a host pass by, the tick try to climb aboard (Arthur, 1962; Dantas-Torres, 2010). *R. sanguineus* can attach to every part of the

dog's body, but their preferred attachment sites are the head (especially on the ears), interdigital spaces, back, inguinal region, and axilla (Dantas-Torres and Otranto, 2011; Louly et al., 2007; Silveira et al., 2009).

According to the number of hosts it is required to succeed its life cycle, ticks are classified into three categories: one-host, two-host or three-host ticks (Pathak, 1987). In the one-host case, all the three instars ticks engorge on the same animal and ecdysis also takes place on the same host (Gray et al., 2013; Pathak, 1987). Most species within *R. sanguineus* group are three-host ticks, characterized by requiring a different host for every life stage (Gray et al., 2013; Pathak, 1987). Nevertheless, there are some ticks within this group that can exhibit two-host ticks behaviour, in which the nymph and adult forms moult on the ground and seek a new host afterwards (Gray et al., 2013; Pathak, 1987). The consequence of that characteristic is having a considerable range of potential hosts. As a typical three-host tick, *R. sanguineus* spends most of its lifetime in the environment, having a very wide host range, which means that there is ground to PA transmission to less-common hosts species, like humans (Labuda and Nuttall, 2004; Wall and Shearer, 2001).

- **Humans as Hosts**

The likelihood of a host other than a dog being parasitized by *R. sanguineus* might vary proportionally to the tick population. In Europe, the human parasitism by this tick is common especially during summer time (or perhaps may be just the period with more reports), in contrast with south American countries where it is common all year long (Parola et al., 2008). Although it is referred that *R. sanguineus* has a weak affinity towards humans (Dantas-Torres, 2010; Rovery et al., 2008; Santos-Silva et al., 2013; Walker et al., 2000), many reports of human parasitism are available (Bacellar et al., 1999; Bastos et al., 2004; Carpenter et al., 1990; Dantas-torres et al., 2006; Estrada-Peña and Jongejan, 1999; Hemmersbach-Miller et al., 2004; Perez et al., 1996; Serra-Freire, 2010). One plausible explanation may be that an alteration of *R. sanguineus* host-seeking and feeding behaviours was undertaken recently, maybe due to unusual climatic circumstances; and some evidence shows an association between the temperature changes and the modification of ticks' behaviour (Dantas-Torres, 2010; Rovery et al., 2008).

Many risk factors are known to be associated with human parasitism by *R. sanguineus*, some of which are listed as follows: high levels of environmental infestation, having dogs in urban areas (Dantas-torres et al., 2006; Nicholson et al., 2006; Uspensky and Ioffe-Uspensky, 2002), dog ownership, presence of infested dogs indoors, daily contact with dogs (veterinarians, dog owners), (Cunha et al., 2009; Dantas-torres et al., 2006; Hemmersbach-Miller et al., 2004; Louly et al., 2006) and high temperatures (Dantas-Torres, 2010; Hemmersbach-Miller et al., 2004; Rovery et al., 2008).

1.1.3. Basic Morphology

Ticks possess many unusual features that distinguish them from other arthropods, such as the lack of compound eyes, antennae or wings; the presence of four pairs of legs in adulthood and three in larvae stage; their reduced segmentation; and their flattened body shape when unfed (because of the fusion of some body segments) (Sonenshine and Roe, 2014). They are also characterized as parasitiforms with a large body size (2-30mm) and present specialized mouthparts for attachment and blood feeding (Sonenshine and Roe, 2014).

There are 3 major regions in the external anatomy of ticks, which are: the anterior gnathosoma or capitulum, the posterior idiosoma or the body, and the legs (beard by the idiosoma) (Sonenshine and Roe, 2014; Wall and Shearer, 2001). The capitulum contains the base of capitulum (also known as basis capituli) that attaches the structure to the body, the chelicerae (appendages that is present instead of biting mandibles, used for cutting, ripping, and tearing skin), the four-segmented palps (in which each segment is also known as article), and the hypostome with rows of recurved teeth meant to host's skin attachment (see Fig.5) (Sonenshine and Roe, 2014; Walker et al., 2003; Wall and Shearer, 2001). The mouth parts of ixodids are more visible from the dorsal aspect, and the eyes are situated on the lateral margin of scutum and conscutum (Pathak, 1987).

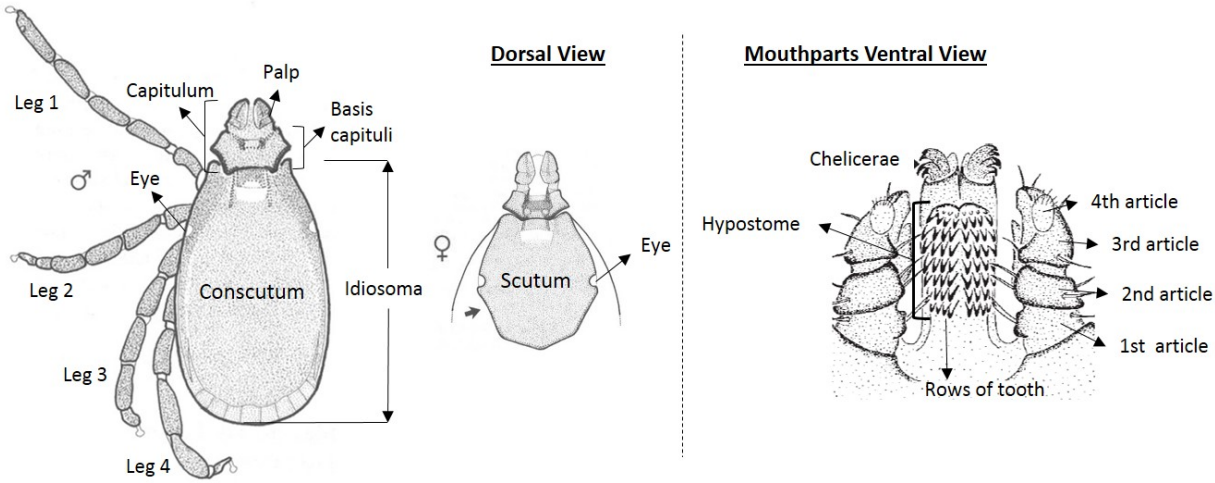

Fig.5 – Basic tick anatomy, adapted from Földvári (2005) and Walker et al. (2003). Adults and nymphs have eight legs. The so-called "head" of a tick includes structures involved in feeding, together known as the "capitulum". It consists of a pair of leg-like sensory structures known as "palps" (divided in four segmented “articles”) that enable the tick to detect an approaching host, a pair of knife-like structures known as "chelicerae" that cut an opening in the host skin, and a single barbed structure known as a "hypostome" that penetrates this skin opening. The hypostome becomes anchored in the host flesh everytime the tick takes a blood meal.

The body is subdivided into an anterior region, the podosoma, bearing the 4 pairs of legs and the genital aperture; and a posterior region, the opisthosoma, bearing the spiracular plate and the anal aperture (see Fig.6) (Sonenshine and Roe, 2014; Wall and Shearer, 2001). The legs are divided into 6 segments (trochanter, femur, patella, tibia, tarsus and pretarsus, as it is identified in Fig.6) and articulate with the body via the coxae (Sonenshine and Roe, 2014; Wall and Shearer, 2001). The coxae may be armed with internal and external ventral spurs, and their number, size and shape are used for the identification of species (see Fig.6, coxae I) (Wall and Shearer, 2001). The tarsus of the first pair of legs contains the Haller’s organ, as can be observed in Fig.6, an important sensory apparatus that includes sensilla (a chemoreceptor setae) for detecting odours, heat, and possible other external factors (Sonenshine and Roe, 2014). The most of chemoreceptors are present on the palps, chelicerae and scutum (Wall and Shearer, 2001).

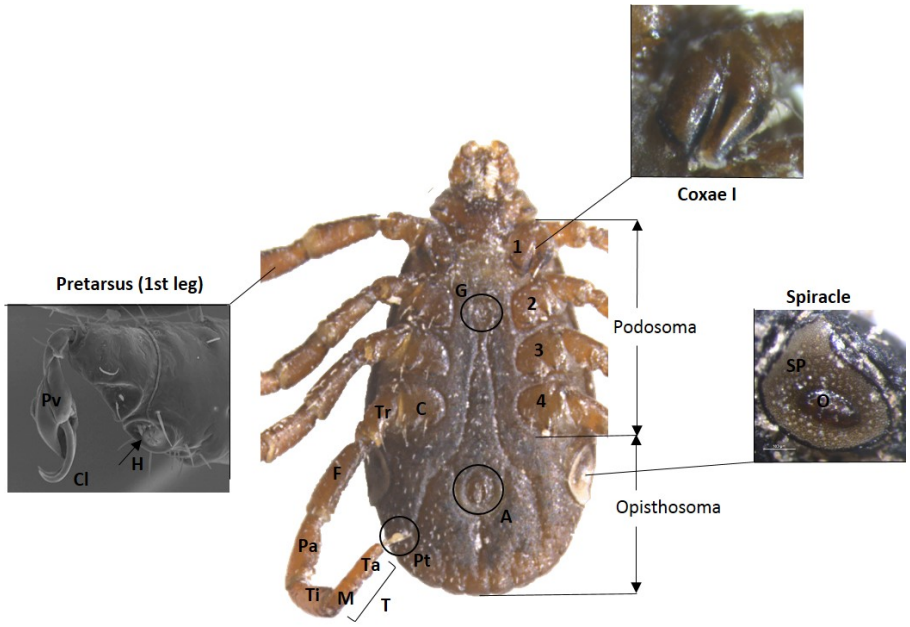

Fig.6 – Ventral view of an unfed female *R. sanguineus*. The tarsus (T) is divided in two subarticles, or tarsomeres: the metatarsus (M) and the tarsus (Ta). The pretarsus, or foot, is pale, delicate and relatively unsclerotized. Its structure is complex and includes two claws (Cl) and an adhesive footpad, or pulvillus (Pv). Haller's organ (H) is a sensory pit located on the dorsum of the first tarsomere of the first leg at the junction with the second tarsomere. It is small but readily discernible with the high power of the dissecting microscope. The first leg has a sensory function used to find and recognize an appropriate host. Note that in coxae I (1) are present two coxae spurs, very typical of *R. sanguineus.* (2) Coxae II, (3) Coxae III, (4) Coxae IV, (A) Anal aperture, (C) Coxae, (F) Femur, (G) Genital aperture, (O) Ostium, (Pa) Patella, (Pt) Pretarsus, (SP) Spiracular Plate, (Ti) Tibia, (Tr) Trochanter.

Male hard ticks (Ixodidae) are usually smaller than females, especially when both are engorged (see Fig.7), and by comparison to soft ticks (Argasidae), possess a sclerotized dorsal shield or plate on the idiosoma known as conscutum (formed by the union of scutum and alloscutum) (Walker et al., 2000; Wall and Shearer, 2001). In the case of females, nymphs, and larvae it constitutes a partial dorsal shield known as scutum (the posterior dorsal part called alloscutum) (Walker et al., 2000; Wall and Shearer, 2001). This explains the limited male's body size growth during feeding, while the females and immature life stages, the body cuticle can expand greatly (see Fig.7), which is accomplished via synthesis of new cuticle rather than simple expansion (Sonenshine and Roe, 2014).

There are also a number of grooves, especially on the dorsal side of ticks, which are very helpful in identification, since they bound several bulges rectangular-like regions in the posterior margins of the body known as festoons (Wall and Shearer, 2001). They are represented in Fig. 8.

For better viewing, both figures (Fig. 7 and Fig. 8) are in the next page.

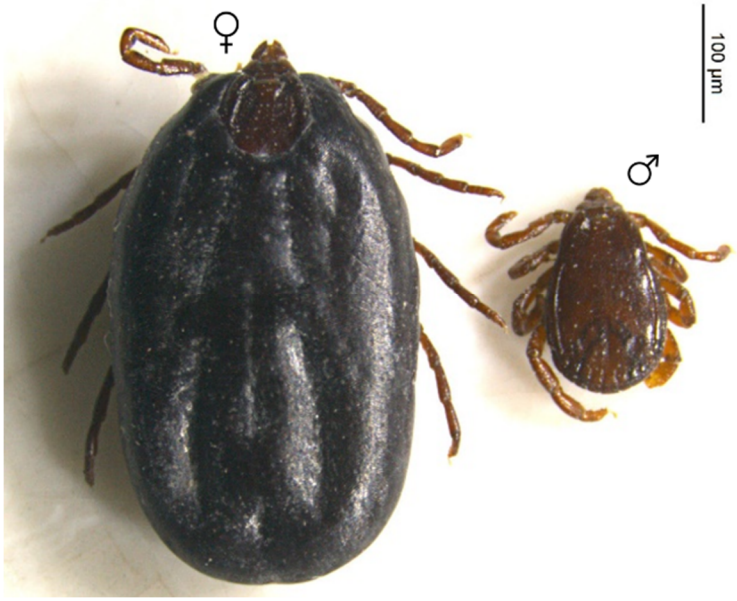

Fig.7 – *R. sanguineus* male and engorged female adults. Note the different in sizes of both *R. sanguineus* genders, due to the extreme expansion capacity of the posterior dorsal part of the female (the alloscutum).

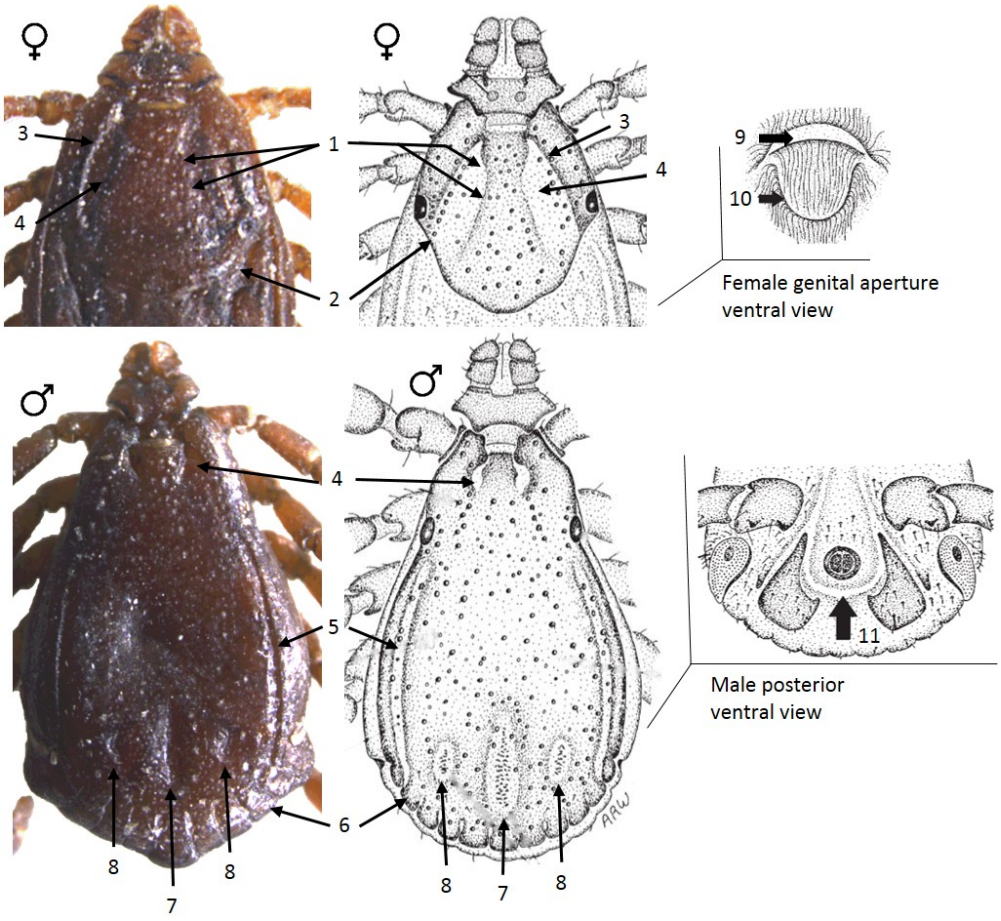

Fig.8 – *R. sanguineus* grooves used in the identification of the species, draws present on the right side were adapted from Walker et al. (2003). Grooves are depressions present in tick scutum, conscutum, around anus and genitalia areas that are useful for family, genus and species identification. Their texture, shape, position and size are also used for identification purposes. (1) Cervical grooves or cervical fields internal grooves; (2) Scutum posterior margin; (3) Cervical fields external grooves; (4) Cervical fields depression, more evident in the female specimen; (5) Lateral grooves; (6) Second festoon; (7) Posteromedian grooves; (8) Paramedian grooves; (9) Genital aperture anterior groove; (10) Genital aperture posterior lips; (11) Anal groove, posterior to anus as is typical in *R. sanguineus*.

When it comes to *R. sanguineus*, we can count 11 festoons in this region (Walker et al., 2003). Spiracles of Ixodida are located near coxae IV in all three families (see Fig.6) and its purpose is to regulate gas exchange and limit water loss from the respiratory system (Pugh and Fordy, 1990 cited in Baker, 1997; Pugh et al., 1988 cited in Baker, 1997; Arthur, 1962). It consists of a spiracular plate, an ostium (see Fig.6), an atrial cavity and atrial muscles (Sonenshine and Roe, 2014). The surface of the spiracular plate is covered with pores called aeropyles and has a macular region that forms the first part of the ostial region of the spiracle (Baker, 1997).

The significantly larger spiracular plate in adult females as compared to males and immature stages, is due not just to overall larger body size, but also to distinct differences in the physiology of females (necessary to the egg production, digestion of larger meals, excretion and other metabolic processes) that increases the cellular metabolism and eventually gas exchange (Baker, 1997). The physiological processes are influenced also by the climate and ecological differences that affect different ticks' populations, affecting in the end various biologic aspects, as the spiracular plate form and size (Baker, 1997).

Sexual differentiation is not obvious in immature stages, which resemble small females without genital aperture, which is situated ventrally behind the gnathosoma (see Fig.6) (Wall and Shearer, 2001). The anus is also located ventrally, usually posterior to the fourth pair of legs, and have the anal groove behind it (see Fig.8) (Wall and Shearer, 2001). The adult males also possess a pair of adanal plates that are lateral to the adanal grooves (for more details see Fig.3 and Fig.6) (Wall and Shearer, 2001).

Particularly, *R. sanguineus* adult ticks may be 3.0 to 4.5mm in length (although size is highly variable and engorged females may reach 12mm); have elongated body shape, short palps and hypostome; the basis capituli is hexagonal dorsally; the coxae I presents two spurs; have eyes and festoons; and presents sexual dimorphism (Walker et al., 2000; Wall and Shearer, 2001). Colourwise, this species can be yellow, reddish or blackish brown and are usually inornate, but the males adanal plates are small and light brown in colour, while the eight-legged nymphs are reddish brown in colour (Wall and Shearer, 2001).

Moreover, the *R. sanguineus* legs may become successively larger from the anterior to the posterior pair, the tarsus of the fourth pair of legs possess a marked dorsal tarsal hook; the anal groove encircles only the posterior half of the anus and then extends into a median

groove (Wall and Shearer, 2001). The punctation of scutum can be defined as fine with sparse larger punctation, and females porose areas are small and their genital aperture are U shaped (see Fig.9) (Földvári, 2005).

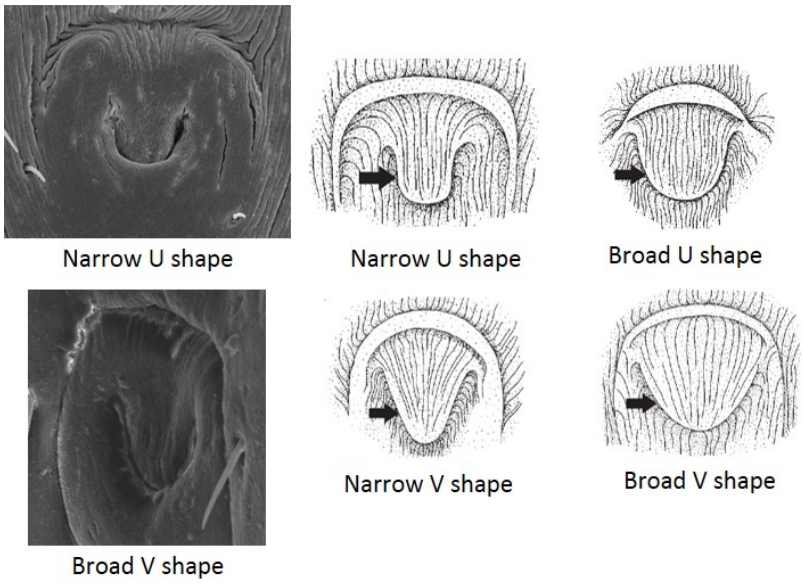

Fig.9 – Genital aperture posterior lips shapes found on *R. sanguineus* and *R. turanicus* species, adapted from Walker et al. (2003). *R. sanguineus* females present U shaped genital apertures, and *R. turanicus* females, in other hand, present V shaped genital apertures. Draws present on the right side of the image were adapt from the cited bibliographic reference.

1.2. Background: Taxonomy and Systematics

On the basis of morphological characters, multiple hypotheses on taxonomic and phylogenetic relationships among hard-ticks taxa have evolved since the middle 19th century (Camicas and Morel, 1977; Hoogstraal and Aeschlimann, 1982; Koch, 1844; Neumann, 1911). Hoogstraal and Aeschlimann (1982) phylogeny is the most frequently cited morphological, biological history and hosts associations based phylogeny among tick families, subfamilies and genera (represented in Fig.10). They suggested that members of Ixodinae arose next to soft ticks and that the metastriate lineage arose more recently.

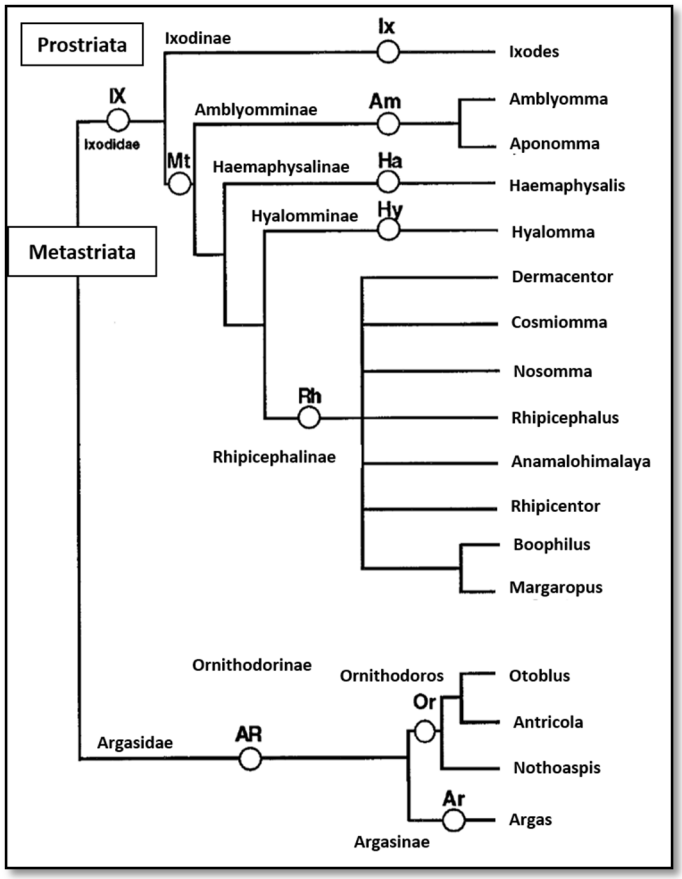

Fig.10 – Phylogeny based on morphology, life history, and host association of ticks families, subfamilies, and genera proposed by Hoogstraal and Aechlimann (1982), adapted from Black et al. (1997). The Ixodidae is split into two major divisions, Prostriata that contains Ixodinae (Ix) and Metastriata (Mt) that contains the remaining 4 subfamilies. IX – Ixodidae, Am – Amblyomminae, Ha – Haemaphysalinae, Hy – Hyalomminae, Rh – Rhipicephalinae, AR – Argasidae, Or – Ornithodorinae, Ar – Argasinae.

Phylogenetic studies based on variation of DNA sequences have challenged that original classification of Rhipicephalinae, and some discrepancies were founded. Phylogenetic analysis using 16S mitochondrial ribosomal DNA (rDNA) reported 5 differences to the traditional classification proposed by Hoogstraal and Aeschlimann (1982): Amblyomminae is not monophyletic, Haemaphysalinae arose within Amblyomminae, Hyalomminae arose within Rhipicephalinae, Argasidae is not monophyletic and Ornithodorinae is bad classified, although there is less information about the latter what means the need for clarification (Black and Piesman, 1994). This conclusions didn't agree with either of traditional classifications (Camicas and Morel, 1977; Filippova, 1966; Hoogstraal and Aeschlimann, 1982). However, the phylogenetic relationships among ticks taxa based on sequence variation in 18S rDNA agree with the Hoogstraal and Aeschlimann (1982) phylogeny, being the only discrepancy the placement of Hyalomminae within Rhipicephalinae (Black et al., 1997), as it is also proposed by 16S

analysis. Their error was to characterize morphological mouthparts features as primitive instead of secondarily derived (Black et al., 1997).

The taxonomy arrangement accepted today says the *R. sanguineus* ticks form the suborder Ixodida in the order Parasitiformes of the subclass Acari (Arthropoda: Chelicerata: Arachnida), where the mites and ticks are grouped (Black and Roehrdanz, 1998).

Nowadays, around 898 ticks' species (Ixodida) subdivided in 3 families are recognized worldwide: the Nuttalliellidae, that includes only one species restricted to southern Africa; the Argasidae or the soft ticks; and the Ixodidae or the hard ticks (including two new recent discovered *Rhipicephalus* spp.) (Apanaskevich et al., 2013; Guglielmone et al., 2010; Horak et al., 2013; Sonenshine and Roe, 2014). The family Ixodidae, the one with wider human and animal health concerns, comprises around 704 species in 14 genera, among which the *Rhipicephalus* are one of the three more numerous groups, with around 84 species (Apanaskevich et al., 2013; Guglielmone et al., 2010; Horak et al., 2013). This genus can be also divided into two ticks-lineages, the prostriate (genus *Ixodes*: Ixodinae) and metastriate (the other four subfamilies, including the genus *Rhipicephalus*) (Barker and Murrell, 2004; Black and Roehrdanz, 1998; Sonenshine and Roe, 2014).

1.2.1. Historical Perspective

Arthropods are among the most ancient animals, first appearing in the Precambrian period 600 million years ago, and they are also considered the most diverse of all the animal phyla in our planet (Raven et al., 2011). Historically, one of the first known references to ticks (Acari: Arachida: Arthropoda) as parasites were made in the famous *Historia Animalium*, written by Aristotle (385-322 B.C.), but the first mention of its ability to harm human beings by transmitting disease was made by M. Porcius Cato, in 200 B.C. (Arthur, 1962). Ticks have been recognized as vectors of disease since 1893, when Smith and Kilbourne discovered *Boophilus annulatus* (=*R. "Boophilus" annulatus*) as an agent carrier of Texas fever in cattle, and the role of ticks in the transmission of viruses, reckettsiae and other organisms have been be studied ever since (Pathak, 1987).

Probably the oldest tick record on an animal species was a recently found tick on a dog mummy of the Ancient Egypt, more precisely in an archaeological site surrounding a Roman fortress in El Deir, located 30 km northeast of the town of Kharga Oasis, Egypt

(Otranto et al., 2014). The ticks-species was identified by Otranto and its colleagues as belonging to the *R. sanguineus* group (established by grouping species whose morphologic similarity between them have led to misidentification) which takes part within the genus *Rhipicephalus* (explained in more detail below) (Otranto et al., 2014). This is another proof that dogs have always been alongside humans as domestic animals, representing the most common pet for humankind throughout our natural history (Otranto et al., 2014; Pegram et al., 1987a). And thus it is normal that this coexistence contributes to a shared zoonotic parasitism history (Otranto et al., 2014).

The taxonomic problem posed by *R. sanguineus* species, and why they are grouped by their similarities, starts by the fact that not much is known about their origin. Some authors believe its origin remounts to African species (Hoogstraal and Aeschlimann, 1982; Walker et al., 2000), while others think it comes originally from Mediterranean species (Morel and Vassiliades, 1962).

Either way, as the genus *Rhipicephalus* is a typical African one (Walker et al., 2000), the hypothesis that *R. sanguineus* have African origin is the most widely accepted.

The *R. sanguineus s.s.* was initially described as *Ixodes sanguineus* by Latreille (1806), but later it was transferred to the genus *Rhipicephalus*. Many authors have described different species under the *R. sanguineus* designation since its original description, some of which have latter been identified as the same species, others put as *R. sanguineus* subspecies (within *R. sanguineus* group), and others even misidentified, specially by Neumann and Zumpt major revisions (Arthur, 1962; Camicas et al., 1998; Gray et al., 2013; Neumann, 1911; Papadopoulos et al., 1992; Pegram et al., 1987a).

Later on, Pomerantsev and its colleagues attributed the *"sanguineus"* reference to the *Rhipicephalus* ixodids found on dogs in the Mediterranean region, and thereafter many of this species *R. sanguineus*-like descriptions tried to follow this new systematic base (Arthur, 1962; Gray et al., 2013; Pegram et al., 1987a; Sonenshine and Roe, 2014).

However, the big problem about all *R. sanguineus* species revisions persisted, which is the lack of the original described type-specimen and its' unspecific description, what created a major identification misunderstanding and disagreement (Gray et al., 2013; Pegram et al., 1987a).

Due to this lack of information, around one quarter of the known species of the genus *Rhipicephalus* were subdivided in eight groups or complexes for ease the study, according to morphological features similarities: *R. appendiculatus, R. cliffordi-senegalensis, R. evertsi, R. kochi, R. pravus, R. sanguineus, R. simus,* and *R. tricuspis* (Camicas et al., 1998; Soares, 2008). In spite of this, each group encompassed a number of specimens with a tremendous "intra-specific" morphologic variability, being the *R. sanguineus* group one of those where this is most evident. This lead to several proposals of different classifications over the years, none of which have definitely closed the controversy around it (Beati and Keirans, 2001; Camicas et al., 1998; Gray et al., 2013; Neumann, 1911; Pegram et al., 1987a, 1987b; Soares, 2008; Walker et al., 2000).

1.2.2. *R. sanguineus* group Issue

The contemporary concept of the *R. sanguineus* group was based on the pioneer ideas of Russian authors (such as Pomerantzev in 1940), but further developed and crafted by Feldman-Muhsam (1956), Hoogstraal (1982), Morel and Vassiliades (1962), and Filippova (Gray et al., 2013; Zahler et al., 1997). Although the type-specimen *R. sanguineus s.s.* has been lost and its origin and description are too poor, as mentioned before, this species still remain the type of the genus and the baseline for this group (Latreille, 1806; Papadopoulos et al., 1992; Pegram et al., 1987a; Soares, 2008).

The taxonomic classification of *R. sanguineus s.l.* is still an ongoing debate, and different approaches can be applied. Currently, at least 11 species are considered within *R. sanguineus* group, namely: *R. sanguineus s.s.* (Latreille, 1806), *R. bergeoni* (described by Morel and Balis in 1976), *R. camicasi* (described by Morel, Mouchet and Rodhain in 1976), *R. guilhoni* (described by Morel and Vassiliades in 1963), *R. leporis* (described by Pomerantsev in 1946), *R. moucheti* (described by Morel in 1964), *R. pumilio* (described by Schulze in 1935), *R. pusillus* (described by Gil Collado in 1938), *R. schulzei* (described by Olenev in 1929), *R. sulcatus* (described by Neumann in 1908), and *R. turanicus* (described by Pomerantsev in 1936) (Gray et al., 2013).

However, it is noteworthy that some authors can consider that *R. aurantiacus* (described by Neumann in 1907), *R. boueti* (described by Morel in 1957), *R. ramachandrai* (described by Dhanda in 1966), *R. rossicus* (described by Yakimov and Kol-Ykimova in

1911), *R. tetracornus* (described by Kitaoka and Suzuki in 1983), and *R. ziemanni* (described by Neumann in 1904) as valid species and included them in *R. sanguineus* group as well (Camicas et al., 1998; Dantas-Torres et al., 2013).

Much of the confusion within *R. sanguineus* group stems from the assumption that *R. turanicus* does not occur in Afrotropical faunal region (based on Pomerantsev new systematic base), and also from the apparent difficulties in distinguishing this species from *R. camicasi*, *R. sanguineus s.s.*, and some morphological variations of *R. sulcatus* (the genital aperture of the common form of this species was reported as indistinguishable from the same morphological structure of *R. turanicus*) (Arthur, 1962; Gray et al., 2013; Pegram et al., 1987a; Sonenshine and Roe, 2014). However, and according to Pegram and colleagues, after adding biological and geographical data to the analysis, all the referred species can be classified as valid (Pegram et al., 1987a)

Pegram and its colleagues (1987b) also described the commons features that specimens needed to present to be considered part of the *R. sanguineus* group. The general morphological characteristics are described below:

- **Male.** Coxal process not visible dorsally. Eyes flat, not furrowed; may be surrounded by a few large punctations. Variable lateral grooves but usually marked out with punctations. Marginal grooves usually deep and containing large, deep punctations. Posteromedian and paramedian wide grooves, fairly short but always distinct. Four almost regular rows of large, deep, proliferous punctations running from the level of the eye to the usually distinguishable posterior grooves (known as "*simus*" pattern). Interstitial punctations variable in size and density. Ventrally, variable spiracular plates (the most useful diagnostic character); adanal plates usually twice as long as it is wide (but it is far too variable intraspecifically to be of diagnostic value, except to *R. sulcatus* and *R. bergeoni*) (Pegram et al., 1987b).
- **Female.** Scutum is usually longer than it is wide. Variable scutum punctation as in males; overall density often appears comparatively greater though. Pronounced lateral grooves and outlined with large punctations. Cervical areas more densely punctate but rare shagreening (except in *R. bergeoni*). Ventrally, the genital aperture is the most valuable diagnostic feature. Variable spiracular plates (except larger in *R. guilhoni*) (Pegram et al., 1987b).

R. sanguineus intra-specific morphological variations have been reported in the literature since its' first description, and although both are recognized as a valid species worldwide, morphological similarity between *R. sanguineus* and *R. turanicus* make them easy to be confused, especially in some populations, as in Portugal (Estrada-Peña and Sánchez, 1988; Pegram et al., 1987a). The only present species of this group in Portugal are *R. sanguineus* (not clearly defined yet), *R. turanicus* and *R. pusillus* (Santos-Silva et al., 2006), and the validation of *R. turanicus* Portuguese populations is still under discussion (Rosa et al., 2013; Santos-Silva, 2010).

▪ Morphological Specificities of *R. sanguineus* and *R. turanicus*

Hoogstraal in 1956, supported by Morel and Vassiliades (1962), mentioned the existence of two *R. sanguineus* ticks "strains", one peridomestic and closely related to dog (with endophilic behaviour), and a "wild race" that also parasitized carnivorous animals (Gray et al., 2013)

Feldman-Muhsam (1956) demonstrated a way to distinguish *R. turanicus* (= *R. secundus* in her work) from *R. sanguineus*, based on morphological examination of females genital aperture (see Fig.11), emphasizing that they can be separated in two distinct entities (Feldman-Muhsam, 1956; Pegram et al., 1987a). According to Feldman-Muhsam (1956), *R. sanguineus s.s.* present a genitalia aperture with "a wider than deep cup", and an circular anterior edge.

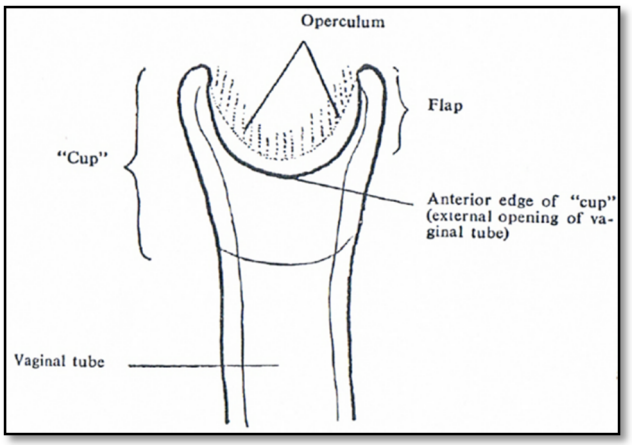

Fig.11 – Schematic diagram of a microscopical examination of a cleared, dissected and mounted adult-female genital aperture of an Ixodid tick, adapted from Feldman-Muhsam (1956). In the image are represented the cup, which form can be more round or more angulate; the flaps, that are the lateral aspects (sclerites) of the cup, that can vary in form, being long or short, rounder or angulate; and the vaginal tube or the stem. The form of the cup and the flaps are specific in different specimens. In *R. sanguineus s.s*, the genitalia aperture has "a wider than deep cup" and a circular anterior edge.

In a study on the biosystematics of the *R. sanguineus* group, Pegram and colleagues initiated a morphological comparative study using SEM images of some of the group species originated from Africa (Pegram et al., 1987a, 1987b). They classified the *R. turanicus* as a species that occur throughout the Afrotropical region in a wide range of climatic biotopes, as well as in parts of southern Europe, Arabia, and Asia, being most abundant in the late rainy-early dry seasons; and it was prone to domesticated and wild animals hosts alike, including ground-feeding birds (Pegram et al., 1987a).

R. sanguineus and *R. turanicus* images are shown in Fig.12 and Fig.13, and this two species were differentiated by analysis of their genital apertures, adanal plates and spiracular plates (Pegram et al., 1987a). Note the differences in the cervical grooves of the males, which are longer on *R. sanguineus*; the final forms of the scutum of females being more linear in *R. sanguineus*; the shape difference of spiracular plates mainly in males (the tail of the spiracular plates are thinner in *R. sanguineus* males); the rounded adanal plate end in *R. sanguineus* when compared with the sharp adanal plate end of *R. turanicus*; and the V-shaped genital aperture of *R. turanicus* compared with the broad U-shaped genital aperture of *R. sanguineus*.

Both figures (Fig.12 and Fig.13) are shown in the next page.

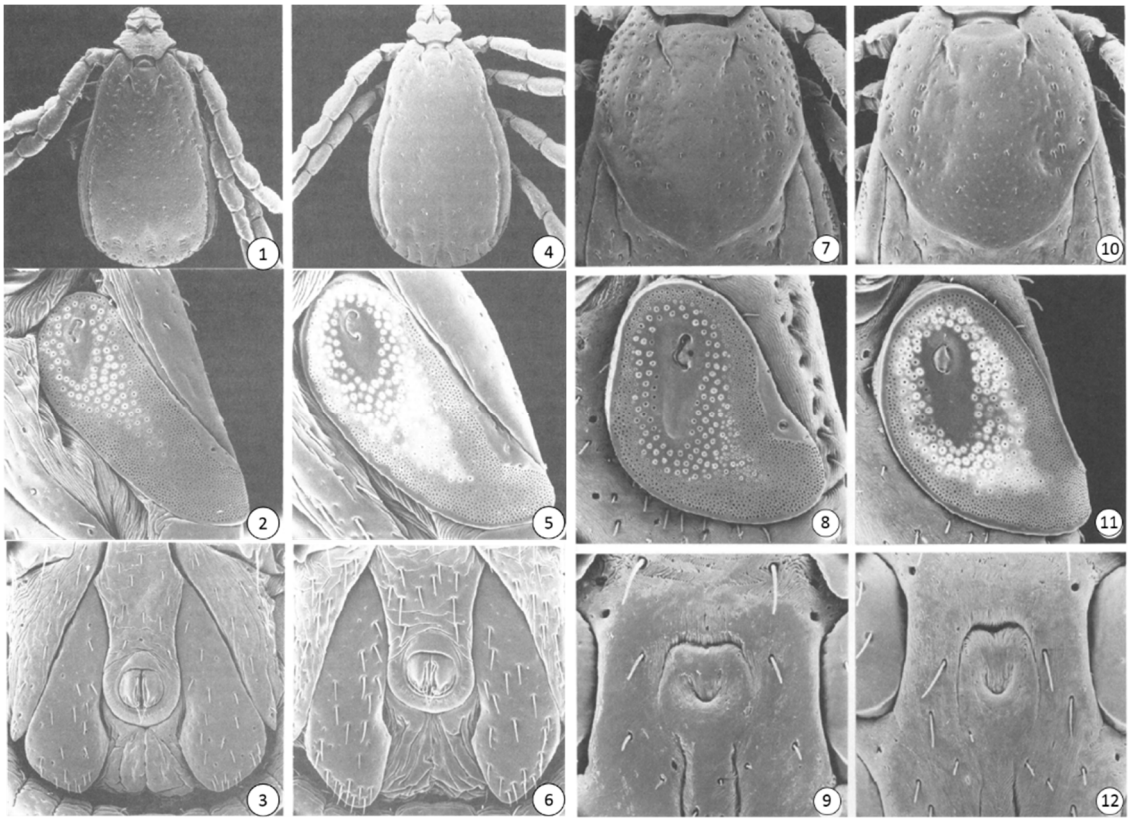

Fig.12 – SEM of *Rhipicephalus turanicus* from Zambia, adapted from Pegram (1987a). Male (1-6): **(**1) and (4), dorsal view; (2) and (5), spiracular plates; (3) and (6), adanal plates. Note the broad shapes on (2) and (5); the square form of (3) and the triangular form of (6). Female (7-12): (7) and (10), scutums; (8) and (11), spiracular plates; (9) and (12), genital apertures.

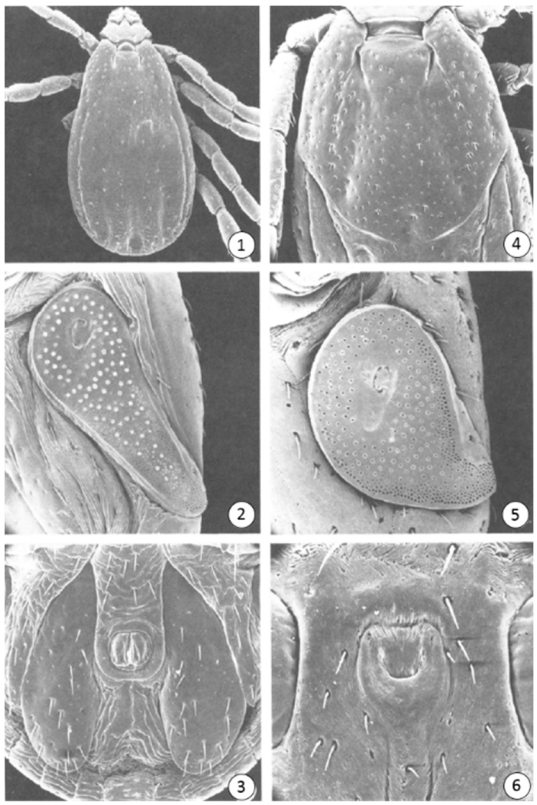

Fig.13 – SEM of *Rhipicephalus sanguineus* from Zambia, adapted from Pegram (1987b). Male (1-3): **(**1) dorsal view; (2) spiracular plate; (3) adanal plates. Female (4-6): (4) scutum; (5) spiracular plate; (6) genital aperture.

They also noted that *R. sanguineus* and *R. turanicus* had many variability in western palaearctic region, but as for DNA features this species are more similar between each other than other reported different species (Pegram et al., 1987a). All of a sudden, the doubt rained down on this matter, revealing all the uncertainties pointed out by previous sequences identified under this names.

Latter, Estrada-Peña and Sanchez (1988) conducted further studies with the aim of finding good taxonomic indicators, by comparison, from some anatomical structures. For that, they used seven morphologic traits: spiracular plate, coxal spines and second palp segment for both genders; adanal plates and parma for male specimens; genital aperture and porose areas separation distances for female. To evaluate whether the correlation between the morphological traits and the species identification is significant, the authors resorted to the chi-square statistical test and to the correspondence factorial analysis. They found that the most differentiating morphological traits for this two species are the spiracular plates; for female, the genital aperture; and for male, the adanal plates. This anatomical structures are depicted in the Fig.14.

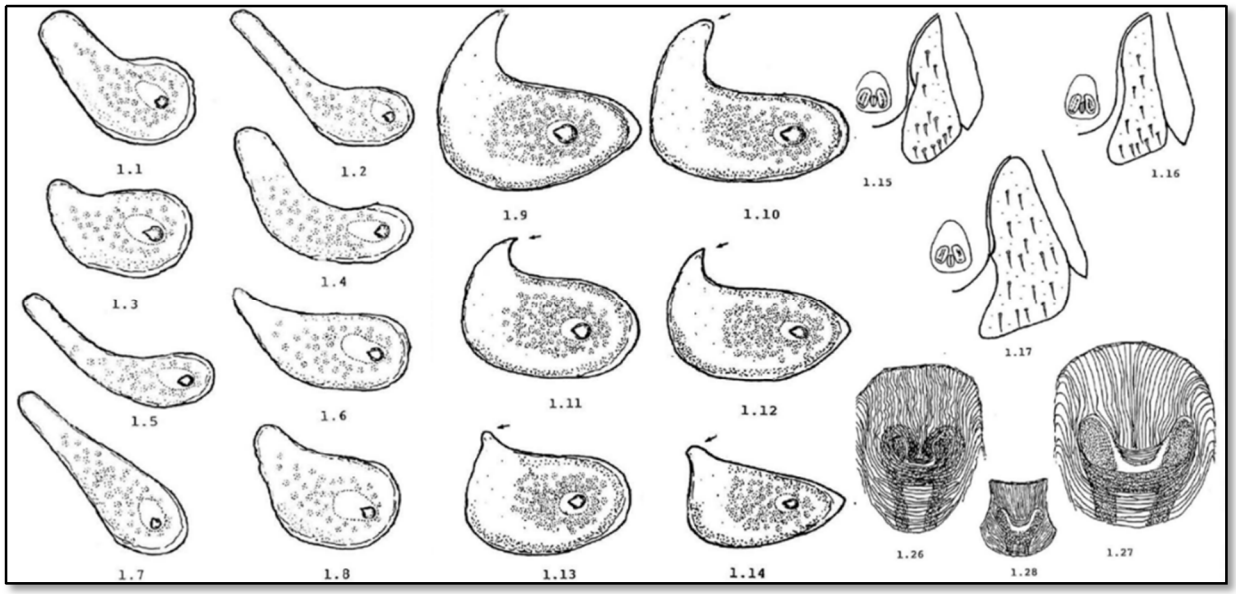

Fig.14 – Schematic diagram of some morphological features found to be the most useful to differentiate the *R. sanguineus* and *R. turanicus*, adapted from Estrada-Peña and Sánchez (1988). Morphological feature: Males spiracular plates (1.1-1.8); Female spiracular plates (1.9-1.14); Male adanal plates (1.15-1-17); Female genital aperture (1.26-1.28). Male spiracular plates with long and narrow tail (1.2) are associated to *R. sanguineus*; and the ones with short and broad tail (1.3, 1.6 and 1.8) are associated to *R. turanicus*. Female spiracular plates with more triangular form (1.12 and 1.14) are *R. sanguineus*; and the ones with more rounded form and broader tale are *R. turanicus* (1.11 and 1.13). Male adanal plates with a final intern angle more rounded (1.15) are associated to *R. sanguineus*, and with a final intern-angle more sharp (1.17) are associated to *R. turanicus.* Female genital aperture with a more rounded or V form (1.27 and 1.28) are from *R. sanguineus* species, being the represented structures only differentiated by the sclerites size; and with a more squared or U form (1.26) are *R. turanicus.*

All the other morphological features were not suitable to distinguish the *R. sanguineus* and *R. turanicus* species (Estrada-Peña and Sánchez, 1988).

It is to noteworthy that, alone, this morphological traits are subjective, being necessary to correlate more than one of them in order to obtain good diagnostic accuracy (Estrada-Peña and Sánchez, 1988).

For the overlapping traits present in both species, the author suggest the possibility of hybridization, what is tuned with some early reports of interbreeding between this two species (Paperna and Giladi, 1974 cited in Estrada-Peña and Sánchez, 1988; Pervomaiskii, 1954 cited in Pegram et al.,1987a.).

In 1992, Papadopoulos and its colleagues also used the spiracular plate and the genital aperture as morphologic indicators (Papadopoulos et al., 1992). The shape of the spiracular plate of *R. turanicus* (Fig.15-1a) is described as a rather short tailed, often angled, and its end width is similar to the adjacent festoon; that of *R. sanguineus* is usually a long tapering tail, whose end width is equal or sub equal to half the width of the adjacent festoon (Fig.15-1b) (Papadopoulos et al., 1992). The females, for other hand, are distinguished by the genital apertures (Fig.15-2): in *R. turanicus* the genital aperture has broad and well pigmented internal flaps, which form a U-shape (Fig.15-2a); in the case of *R. sanguineus*, the internal flaps are semicircular and lightly pigmented (Fig.15-2b) (Papadopoulos et al., 1992), what could be described as a V-shape.

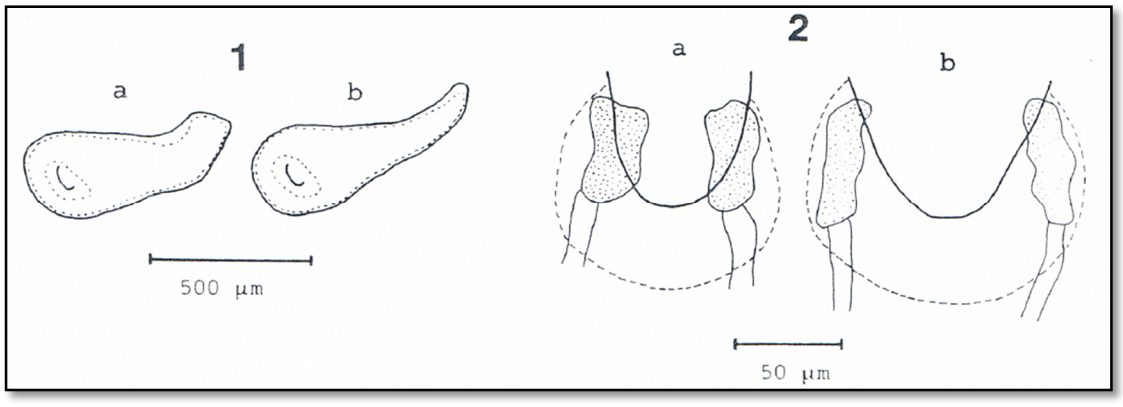

Fig.15 – Schematic diagram of spiracular plates and genital apertures of *R. turanicus* (a) and *R. sanguineus* (b) adapted from Papadopoulos et al. (1992). 1. Representation of a spiracular plate of male of *R. turanicus* (a) and *R. sanguineus* (b). 2. Representation of genital apertures of females of *R. turanicus* (a) and *R. sanguineus* (b)**.** Note the short and angled tale of *R. turanicus* compared with the long tapered tale of the *R. sanguineus*; as well as the U-shape of the *R. turanicus* female compared to the V-shape of the *R. sanguineus* female.

Although immature forms have smaller morphological variability, Russian authors consider both *R. turanicus* and *R. sanguineus* valid species based on the analysis of this species immature forms (Gray et al., 2013).

Despite some consensus in what regards the morphological variability studies on these two species, some contradictory results do exist.

For example, in Sanchez and colleagues studies (1992), they compared different spiracular plates morphologies obtained in Spanish *R. sanguineus* and *R. turanicus,* and concluded that the females of both species in the country were easily distinguished by the correct evaluation of the referred morphological trait. However they came across other conclusions, of note the display of high phenotypic variability in this species (Sanchez-Acedo et al., 1992). This might be explained by the fact that the work of Feldman-Musham (1956) were biased by the low number of specimens studied as well as their origin, because all the specimens were only collected from dogs found in different regions. In conclusion, this might mean that the Feldman-Musham conclusion may not be universally reproducible due to the wide morphological variability of this ticks (Gray et al., 2013; Pegram et al., 1987a).

The intra-specific variations of the *R. sanguineus* morphology previously described can be associated with many factors, such as the study's population group location, preservation of the method used, engorgement level in the case of the females, observation angle, personal interpretation or even morphological structures overlapping caused by hybridization of similar species (Estrada-Peña and Sánchez, 1988; Pegram et al., 1987a; Ribeiro et al., 1996). Some authors even suggested that *R. sanguineus* and *R. turanicus* have a close genetic relationship compatible with possible conspecificities, that is, they can represent a single species, based on molecular markers analysis (ITS2,12S and 16S mitochondrial rDNA) (Beati and Keirans, 2001; Mangold et al., 1998; Santos-Silva, 2010; Zahler et al., 1997).

However, this results did not agree with previous studies conducted with Israel *R. turanicus* populations (Black and Piesman, 1994; Ioffe-Uspensky et al., 1997), what may mean that different geographical zones influence different tick populations in variable evolution directions, leading to more or less genetic diversion between these two species. Other explanations relate this phenomenon, as referred before, with the capacity of *R. turanicus* to hybridize with some *R. sanguineus* group species (Beati and Keirans, 2001;

Estrada-Peña and Sánchez, 1988; Pegram et al., 1987a; Ribeiro et al., 1996), or even that this species recently diverged within the genus *Rhipicephalus* (Mangold et al., 1998).

Overall, local and regional variations of this species are very common, what leads us to the discussion on whether it is possible to define a boundary separating this two "groups" as different species or not, a question still calling for a conclusive answer.

1.3. *R. sanguineus*: Updated Knowledge

Even today it is not completely clear whether all ticks under the *R. sanguineus* name, collected worldwide on carnivorous hosts, represents a single species (Estrada-Peña and Sánchez, 1988; Walker et al., 2003). A paradigmatic example is the presence of two sympatric members of the *R. sanguineus* group in Europe – *R. sanguineus* and *R. turanicus* – that can be easily misidentified, because of the tremendous similarity between them (Estrada-Peña and Sánchez, 1988; Walker et al., 2003). Accurate identification of ticks species is important to control TBD (Sonenshine and Roe, 2014; Szabó et al., 2005), and combining molecular data from different genes and morphological features may help to solve this unclear taxonomic relationships.

1.3.1. Disease Vector Role and Health Significance

Although mosquitoes transmit pathogens that infect a greater number of hosts and cause more severe diseases in both animals and humans (such as malaria, dengue fever or yellow fever) (Lemon et al., 2008), ticks transmit a greater variety of PA than any other disease-vector (Dantas-Torres, 2008; Jongejan and Uilenberg, 2004).

As hematophagous arthropods and due to a number of biologic attributes that enhance their vector potential (described in Table.1), ticks are often vectors of several disease agents, such as viruses, bacteria, rickettsiae, protozoa and fungi, that can affect many tick hosts, being the *R. sanguineus* a dog parasite with a high vector capacity (Dantas-Torres, 2010; Földvári, 2005; Rosa et al., 2006; Walker et al., 2000).

Table.1 - Essential biological characteristics of ticks as vectors of Pathogenic Agents, adapted from Santos-Silva et al. (2006) and Sonenshine and Roe (2014). PA – pathogenic agents.

Essential Characteristics	Justification
Obligatory hematophagous	Sucking blood parasite, that allow transmission of PA by saliva, infected hypostome, infected host's blood, and others
Three biological cycles: mono-, di-and three-phase life, depending on the use of 1, 2 or 3 hosts	Diphase cycle: one host to the immature phases, one to the mature. Triphase cycle: one host to witch phase, more PA transmission occur
Feeding is a slow process	Provides an extended period of interaction
Ingestion of large volumes of blood	Greater amount and time necessary to feed, more probability of PA transmission
Gradual, intracellular and without enzymes **digestion**	PA do not die easily in the digestion
Transmission occurs via **transtadial** or horizontally, and **transovarian** or vertically	*Transtadial Transmission* - PA survive to the metamorphosis, remains with the vector from one life stage to the next; *Transovarian Transmission* - PA are transmited to the progeniture, being then called a natural reservoir. Important for PA maintenance
Sensory system developed	Tracking and finding the hosts easily
Diapause	*Adaptive feature* - capacity of change the duration of the different phases of development to better survive in difficult environment
Great longevity and high rates of **prolificacy**	More probability to transmit PA
Reduced number of **predators**	Less probability to die
Distribution in almost **all habitats**	More probability to transmit PA

This arthropods can transmit pathogens mostly through the feeding process, for what it is necessary the insertion of the feeding tube in the host skin (Arthur, 1962). Its own saliva, which have anaesthetic properties, can be the source of infection (Arthur, 1962; Pathak, 1987).

For a species to be effective as a vector, factors such as population density, species longevity, postures' number and feeding behaviours have a huge impact on the transmission of disease, due to variation of vectors capacity (ability of certain species to transmit the pathogen in spatial and temporal terms) and vectors competence (ability of biological maintainability of infection, which consequently enables its transmission) (WHO, 1985). Probably around 10% of the acknowledged species of ticks are implicated in the transmission of PA (Arthur, 1962), in which *R. sanguineus* is included. It is important to emphasize that the prevalence of *R. sanguineus* pathogens can change biogeographically (Parola and Raoult, 2001; Szabó et al., 2005).

It is to note that all of the tick-borne infectious diseases are zoonoses, which means that are animal diseases transmissible to humans, so dogs as human pets favour the most part of human infections caused by this diseases (Rosa et al., 2006; Sonenshine and Roe, 2014). Although the extraordinary development in Medicine and pest control regarding

this vector-borne diseases, as far as the scientific community knows, these pathogens have persisted and some can even increase their geographic range in the next years (Oliveira et al., 2012; Parola and Raoult, 2001; Rodríguez-Mallon et al., 2012; Samish et al., 2004; Sonenshine and Roe, 2014; Thangamani and Bente, 2014).

- **Pathogenic Diseases Associated**

R. sanguineus is the principal vector of *Rickettsia conorii*, the agent of canine rickettsiosis in dogs and Boutonneuse fever in humans (spotted fever and Israeli tick typhus) present in the countries around the Mediterranean coast (Bacellar et al., 1999; Estrada-Peña and Jongejan, 1999; Jongejan and Uilenberg, 2004; Psaroulaki et al., 2003; Renvoisé et al., 2012; Uspensky and Ioffe-Uspensky, 2002). The most important canine diseases transmitted by this tick are babesiosis, caused by *Babesia canis*, and monocytic ehrlichiosis, caused by *Ehrlichia canis* (Bastos et al., 2004; Gray et al., 2013), although *R. sanguineus* ticks are able to transmit more pathogens, as shown in Table.2 (pg.30).

In addition, tick bites can lead to severe toxic reactions, allergic responses, or even deadly paralytic symptoms (tick paralysis) (Sonenshine and Roe, 2014).

R. sanguineus is one of the species that more frequently parasitize humans, and there are several records of human bitten by this tick, namely in Argentina, Brazil, Chile, Mexico (southern region), Panama, Peru, Puerto Rico, Venezuela, Uruguay, United States of America (USA), and Mediterranean countries including Portugal (Bacellar et al., 1999; Bastos et al., 2004; Carpenter et al., 1990; Dantas-torres et al., 2006; Estrada-Peña and Jongejan, 1999; Hemmersbach-Miller et al., 2004; Perez et al., 1996; Serra-Freire, 2010).

The two most important human pathogens transmitted by *R. sanguineus* are *Ri. conorii*, the cause of Mediterranean spotted fever (or Boutonneuse fever) especially in countries around the Mediterranean littoral, and *Ri. rickettsii*, which is the etiological agent of Rocky Mountain spotted fever specially in USA (Dantas-Torres, 2008; Dantas-Torres et al., 2012; Nicholson et al., 2006; Parola et al., 2009, 2005; Sonenshine and Roe, 2014). All of these TBD may cause significant morbidity, or even mortality (Gray et al., 2013) if not treated properly.

Table.2 - Etiologic agents of some canine diseases that are transmitted or have high probability to be transmitted by *R. sanguineus* ticks, and their geographic distribution (Bacellar et al., 1995; Bastos et al., 2004; Cardoso et al., 2010, 2008; Claerebout et al., 2013; Cunha et al., 2009; Dantas-Torres, 2010; Dantas-Torres et al., 2011; Demma et al., 2006; Földvári, 2005; França et al., 2010; Jongejan and Uilenberg, 2004; Márquez et al., 2008; Ndip et al., 2010; Olmeda-García et al., 1993; Otranto et al., 2012; Psaroulaki et al., 2003; Zahler et al., 2000)**.**

Pathogenic Agents	Disease Associated	Geographic Distribution	Reference
Anaplasma platys	Canine cyclic thrombocytopenia	Africa, Europe	Cardoso et al, 2008
Babesia canis canis	Canine babesiosis	Tropical and semitropical regions	Bastos et al, 2004; Cardoso et al, 2010; Zahler et al, 2000
Babesia canis vogeli	Canine babesiosis	Tropical and semitropical regions	Jongejan and Uilenberg 2004; Zahler et al, 2000
Babesia gibsoni	Canine babesiosis	Africa, Asia, USA, southern Europe, Middle East	Zahler et al, 2000
Cercopithifilaria grassi	Canine filariosis	Mediterranean, USA, Brazil, eastern Asia	Otranto et al, 2012
Cercopithifilaria bainae	Not defined	Mediterranean	Ramos et al, 2014
Dipetalonema dracunculoides	Canine filariosis	Mediterranean, Switzerland	Olmeda-García et al, 1993
Ehrlichia canis	Canine monocytic ehrlichiosis	Southern USA, Europe, Africa, Middle East, eastern Asia	Cardoso et al, 2010; Foldvári, 2005; Jongejan and Uilenberg 2004; Ndip et al, 2010; Zahler et al, 2000
Ehrlichia chaffeensis	Canine babesiosis	Cameroon (Africa)	Ndip et al, 2010
Hepatozoon canis	Canine hepatozoonosis	Southern Europe	Cardoso et al, 2010; Foldvari, 2005; Jongejan and Uilenberg 2004
Leishmania infantum [b]	Canine visceral leishmaniosis	Brazil, Italy	Dantas-Torres et al, 2010b, 2011
Mycoplasma haemocanis	Canine haemobartonellosis	Mediterranean	Kemming et al, 2004
Rangelia vitalli [b]	Nambiuvu or Bleeding Plague	Southern Brazil	França et al, 2010
Rickettsia conorii [a]	Canine rickettsiosis	Southern Europe, Middle East, Africa	Márquez et al, 2008; Psaroulaki et al, 2003
Rickettsia felis	Canine rickettsiosis	North and South America, Eastern Europe, Central Africa, Australia	Cardoso, 2006
Rickettsia massiliae [a]	Canine rickettsiosis	Europe, USA	Claerebout et al, 2013; Márquez et al, 2008; Psaroulaki et al, 2003
Rickettsia rhipicephali	Canine rickettsiosis	USA, Europe	Bacellar et al, 1995
Rickettsia ricketsii	Canine rickettsiosis	Central and South America	Demma et al, 2006, Cunha et al, 2009

[a] identified to be also an pathogenic agent of *R.turanicus*

[b] some evidences indicated *R. sanguineus* as vector, but futher research is needed to prove it.

Mediterranean spotted fever is referred as an emerging or re-emerging disease in some countries, and its incidence reports has substantially increased in the past 10 years in some Mediterranean basin countries (Bacellar et al., 1999; Lemon et al., 2008). This probably happen because the real severity of this disease have been ignored for at least 70 years, due to the medical concept that this disease was benign (Rovery et al., 2008). Probably because of this lack of medical concern towards Mediterranean spotted fever back then, its real severity was underrated for a long time, and once more attention was regain, the number of reports increased, as may have happen in many other diseases described (Lemon et al., 2008; Rovery et al., 2008; Sonenshine and Roe, 2014).

The increasing incidence in the literature of human TBD transmitted by *R. sanguineus* indicates that the tick-human interaction may be more common, and less accidental, than it is recognized in the American and European continents. However, it is also true that human infection incidence can be rising as a result of contacts with contaminated environments, with biological fluids and from handling infected animals (Sonenshine and Roe, 2014; WHO, 1985). Despite being just a hypothesis, in the future the climate changes can be an influence factor in the modification of epidemiologic patterns of both human and animal TBD, perhaps leading to the emergence of new healthcare problems (Földvári, 2005; Lemon et al., 2008).

1.3.2. Phylogenetic Molecular Studies

With the advancement of scientific knowledge, it began to be possible to use molecular biology, specifically genetics and molecular analysis tools, as an aid instrument in the identification of tick species.

The advent of polymerase chain reaction (PCR), by Kary Mullis in 1983, marked the beginning of a revolution in molecular biology, and consequently on systematics, evolution and taxonomy (Lewis, 2008). This method not only speeds up the DNA sequencing, but also facilitates the selection of gene markers, needing only small amounts of molecular material to be performed (Lewis, 2008). DNA markers have since been widely tested ever since, and its utility has been proved handful not only for mites and ticks (Black and Piesman, 1994; Black et al., 1997; Crampton et al., 1996; Murrell et al., 2001, 2000), but also in the study of other arthropods (Cruickshank, 2002).

Employing appropriate genetic markers, in particular mitochondrial DNA (mtDNA) and nuclear markers, has proved to be a valuable complementary tool to the traditional morphological approach, eventually reducing the number of closely-related species misidentification. Moreover, an increased number of recorded mitochondrial genes, and even complete mitochondrial genomes, of tick specimens are being recorded in a reference database facilitating the comparison of its genetic sequences from this arthropods (Beati and Keirans, 2001; Burlini et al., 2010; Erster et al., 2013; Gray et al., 2013; Szabó et al., 2005). Furthermore, these sequences are currently being used to determine phylogenetic relationships between related species, and can be further complemented afterwards with the traditional taxonomic classification based on morphological features (Erster et al., 2013).

Molecular phylogenetic is based on the assumption that the divergence of nucleotide sequences between a pair of genomes should lead to the indication of how long the two genomes have shared a common ancestor (Sonenshine and Roe, 2014), making the choice of the molecular marker or DNA sequence to be used in a particularly study widely important. However, no ideal molecular marker exists (one which guarantees proper and reliable results based on its molecular features).

That been said, an ideal marker should be or have: 1) a *single-copy sequence target or multiple homogenous copies*, to avoid obtainning different copies of the gene from different individuals in the same phylogeny, although the former may be difficult to amplify (an example of a sequence target with multiple homogenous copies are mitochondrial genes and nuclear ribosomal genes); 2) *sequences easy to align*, because gene length can vary between taxa, and so sequences must be aligned before phylogenetic analysis (for example protein-coding genes, once gaps occur in groups of three); 3) *sequence sites equally free to vary*, in as much as only about a third of the sites are free to vary without altering the protein sequence and so only this sites are likely to be phylogenetically useful (at least for closely related taxa); 4) *mutagenic substitution rate* high enough to provide sufficient number of valuable sites, but low enough to avoid excessive substitutions that can mask previous ones, which would increasingly complicate the phylogenetic analysis; 5) *equal base compositions and no variation among taxa*, because otherwise the amount of homoplasy in the data set would increase and some methods of phylogenetic analyses would not work properly (some of these analysis group sequences based on the similarity of base composition), what can be a problem for

mitochondrial genes which tend to be AT rich; 6) *universal primers and sequences used before*, because it is easier to work with primers suitable to a wide range of taxa (they are more likely to be conserved) even though it can lead to amplification of gut contents (Yli-Mattila et al., 2000). Additionally, sequences already used can facilitate the post analysis by comparison and even allow us to map broader scale phylogenies (Cruickshank, 2002).

There for, once genomic sequences have a variety of functions, and so different rates and patterns of nucleotide substitutions, we must be aware that, depending on the study goals and sequences used, different markers should be chosen. Nevertheless, it is also of note that some studies have revealed discrepancies between morphological and molecular taxonomies, as distinct species sometimes cannot be differentiated via molecular methods, as it is the case of some lineages within Rhipicephalinae (Beati and Keirans, 2001; Rosa et al., 2013; Santos-Silva, 2010; Zahler et al., 1997). This particular finding may be due to mitochondrial introgression (purposeful introgression is a long-term process and it may take many hybrid generations before the genetic backcrossing occurs permitting the gene flow to allied species) may be detected (Rees et al., 2003).

In general, nuclear ribosomal gene sequences are more reliable to define the family and subfamily levels (Beati et al., 2008; Black et al., 1997; Dobson and Barker, 1999; Fukunaga et al., 2000; Klompen et al., 2000); whereas mitochondrial genes and internal transcribed spacers (ITS) provide better resolution at the intrageneric and intraspecific levels (Barker, 1998; Beati and Keirans, 2001; Klompen et al., 2000; Szabó et al., 2005).

- **Mitochondrial Markers**

The mitochondrial genome is considered a useful tool to establish relationships between closely related species due to its small size and relatively fast rate of evolution, compared with nuclear genome (Brown et al., 1979; Latrofa et al., 2013; Shao and Barker, 2007). mtDNA sequences are then useful as molecular markers for the identification and differentiation between organisms, particularly for populations and systematic genetics approaches (Beati and Keirans, 2001; Burlini et al., 2010; Dantas-Torres et al., 2013; Erster et al., 2013; Liu et al., 2013; Szabó et al., 2005).

The metazoan mitochondrial genome, ranging in length from 14 to 18 kb approximately, is typically circular an usually contains 36-37 genes, including 12-13 protein-coding

genes, 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes and 2 non-coding control regions (NCR) and some intergenic spacers (Boore, 1999).

Mitochondrial genes occur in large scale in each cell, but usually all of its copies have the same sequence due to the uniparental inheritance of this organelle (Lewis, 2008). This genes fall in two categories: ribosomal genes (12S and 16S rDNA) and protein-coding genes (eg. cytochrome c oxidase I – COI). Their single-copy genes make them easy to work and useful at an intraspecific level, what is due to its tendencies to clear A+T accumulation, to accumulate transversions as the distance between taxa increases, to accumulate substitutions, and it to have a strictly maternal inheritance (Simon et al., 1994). However, some mitochondrial genes can be transposed to the nuclear genome (mitochondrial pseudogenes or nuclear mtDNA – numt) and are known to be found in eukaryotic genomes, such as in arthropods, and as a result, the interpretation of the nuclear genes variation should be done with great caution to not result in some species misidentification (Cruickshank, 2002; Richly and Leister, 2004).

Furthermore, 16S mt rDNA is useful to studies below family level (Black and Piesman, 1994), while 12S rDNA is useful to study intraspecific variations due to its many hypervariable regions, especially in recent speciation events and when in combined studies with other markers (as COI, ITS2 or 18S nuclear rDNA) (Beati and Keirans, 2001; Cruickshank, 2002; Mangold et al., 1998; Murrell et al., 2001, 2000, 1999).

The 5’ region of the mtDNA gene COI (or COX1) is the standard marker to DNA barcoding in all animals (Cruickshank, 2002). COI has a similar range of uses to ITS2 (referred below), but appears to evolve slightly faster, and its sequences are easier to align that 16s rDNA once it is a protein-coding sequence that is, it presents no gaps within the alignment (even though the 16S sequence quality is better), being used in closely related species or even genus levels (Cruickshank, 2002; Lv et al., 2014).

However, attempts to sequence COI in some species often fail probably because the sequence tends to be more variable than previously thought and some rearrangements of the control region can occur (Black and Piesman, 1994; Black et al., 1997; Boore, 1999; Dantas-Torres et al., 2013; Murrell et al., 2000). These observations argue for careful and thorough taxon sampling when using this genes as markers of deep-level phylogenetic relationships among taxa (Black and Roehrdanz, 1998).

- **Nuclear Markers**

18S rDNA, 5.8S rDNA and 28S rDNA nuclear genes are transcribed as a single ribonucleic acid (RNA) transcript separated in the two ITS regions of nuclear ribosomal genes, ITS1 and ITS2, which are non-coding regions and, as so, they are under very low selection pressure and can accumulate substitutions quickly (Cruickshank, 2002). This characteristics can be useful for distinguish between closely related species and for intraspecific variations studies (Cruickshank, 2002). ITS1 has similar proprieties to ITS2, but it is more variable and also more difficult to align (Navajas et al., 1999), what makes of the ITS2 the marker more commonly used.

ITS2 have significantly advanced our understanding on evolution of ticks, but can result in ambiguous assignment of some closely related-species, as *R. sanguineus* and *R. turanicus*, raising concerns on the suitability of at least some loci of this marker to species-level identifications within the *R. sanguineus* group (Barker, 1998; Latrofa et al., 2013; Zahler et al., 1997). The possible causes behind this may be not only the unequal crossing over and gene conversion suffer by this region, what makes it so homogenized even when change is introduced to one of this very closely related species (the latter being proved by past crossbreeding experiments) (Gerbi, 1986; Zahler et al., 1997); but also because *Rhipicephalus* is one of the most difficult genera of ticks to be taxonomically classified due to its high level of morphological intrageneric uniformity and intraspecific variability (Arthur, 1962; Barker, 1998; Beati and Keirans, 2001; Walker et al., 2000).

Similar results have been obtained using different nuclear target gene, such as 18S and 28S rDNA, further indicating that nuclear DNA is unsuitable for molecular discrimination of closely related species of most ixodids ticks, as a consequence of the low genetic distance verified (Anstead et al., 2011; Latrofa et al., 2013). For example, there is a proven lack of resolution within and between Rhipicephalinae and Haemaphysalinae using this genes (Crampton et al., 1996), probably due to their very closely related members. However, this nuclear sequences have been used at deepest taxonomic levels because these regions are more conserved than 16S mitochondrial rDNA, and therefore, less subject to homoplasy (Black et al., 1997; Crampton et al., 1996; Cruickshank, 2002; Dobson and Barker, 1999; Simon et al., 1994).

ITS2 and COI together provide a powerful tool for studies of intraspecific variation and phylogenies of closely related species (Navajas et al., 1998), and 18S rDNA and 28s rDNA are identically useful at the other end of the taxonomic spectrum. Thus, the advances in molecular biology allowed for quicker molecular classification methods, and several studies have been conducted to assess the suitability of molecular markers range as well as the identification degree accuracy of ticks within *R. sanguineus* group (Burlini et al., 2010; Dantas-Torres, 2010; Moraes-Filho et al., 2011; Szabó et al., 2005).

1.3.3. Population Genetics

Over the last decade, many investigators have tried to evaluate the genetic variability of *R. sanguineus s.l.* and, with that information, differentiate closely related data within *R. sanguineus* group from different geographical localities.

The conspecificity of *R. sanguineus* and *R. turanicus* has been suggested based on the results of DNA analysis with ITS2 *R. sanguineus* sequences from Azerbaijan and Burkina Faso, directly compared with the same DNA sequences of *R. turanicus* specimens from Turkmenistan (Zahler et al., 1997). The combined analysis of mitochondrial 12S ribosomal DNA gene sequences with morphological characters of the Mediterranean shore populations of *R. sanguineus* and *R. turanicus* compared with the Turkmenistan population also concluded that this two entities represented a single species (Beati and Keirans, 2001).

For example, when interbred, *R. sanguineus* ticks from Azerbaijan and *R. turanicus* from Turkmenistan produce fertile F1 progeny (Pervomaisky, 1950 in Gray et al., 2013), what constitutes a strong argument for their conspecificity. Moreover, *R. sanguineus* taxon itself seems not to be homogeneous and may represent more than one species, being that 16S and 12S mitochondrial rDNA sequences studies separate the tick specimens from different populations into two distinct clades (Burlini et al., 2010; Levin et al., 2012; Moraes-Filho et al., 2011; Nava et al., 2012; Szabó et al., 2005). Other experiments in interbreeding between ticks from genetically distinct populations have confirmed this separation on the reproductive level (Gray et al., 2013; Szabó et al., 2005)

More recently, a comprehensive study was undertaken on representative ticks specimens belonging to the *R. sanguineus* group from 17 countries (in Europe, Africa, Americas and

Oceania) where morphological (punctation pattern, dorsal scutum shape, spiracular plates shape, adanal plates shape, accessory fields shape, and genital aperture shape) and molecular analysis (cox1, 12S and 16S rDNA gene sequences were analysed) revealed the existence of at least four integrated operational taxonomic units (IOTU) or lineages under the name *R. sanguineus* (Dantas-Torres et al., 2013), such as: *R. sanguineus s.l.*, *Rhipicephalus* sp. I, *Rhipicephalus* sp. II and *Rhipicephalus* sp. III (Dantas-Torres et al., 2013). The reference "*R. sanguineus s.l.*" was then use to cite the *R. sanguineus* African specimen description by Walker (2000) and "*R. turanicus*" reference was based on Filippova (1997) description of the Turkmenistan specie. Ticks that could not be morphologically assigned to this two groups were defined as "*Rhipicephalus* sp" with the respective number of the cluster where the specimens were placed in (I, II, III…) (Dantas-Torres et al., 2013). Their phylogenetic analysis supported the morphological identification, and even separate the *Rhipicephalus* sp. from *R. sanguineus s.l.* and *R. turanicus* specimens (Dantas-Torres et al., 2013)

Dantas-Torres and colleagues based their analysis on some of the crossbreeding studies referred above whose conclusions provided evidence with crossbreeding studies that some taxa currently identified as *R. sanguineus s.l.* may actually represent separated species; and also on some studies that claimed the existence of two different lineages of *R. sanguineus s.l.* species: the tropical species or northern lineage and the temperate species or southern lineage (Dantas-Torres et al., 2013; Levin et al., 2012; Nava et al., 2012; Pegram et al., 1987a; Szabó et al., 2005).

1.4. *R. sanguineus* in Portugal

Portugal, the most western country of continental Europe, have essentially three types of climate: Mediterranean, in the southern zone; Oceanic, along the littoral and northern areas; and Continental in some islands of the northeast and central east region of the country (Estrada-Peña and Santos-Silva, 2005). The occurrence of suitable hosts and favourable climate conditions in this country benefit the distribution, spread and maintenance of ticks and, consequently, of TBD in Portugal (Dias, 1994; Estrada-Peña and Santos-Silva, 2005; Walker et al., 2003).

R. sanguineus is widely distributed in the country (see Fig.16) and can be active all year long, the highest populations densities registered occur essentially on the warm months (Santos-Silva et al., 2013; Szabó et al., 2008). Although, some differences are registered for each region in Portugal (Santos-Silva, 2010; Santos-Silva et al., 2013), as depicted in Fig.16, they can be explained by ticks populations variability and its ecological characteristics.

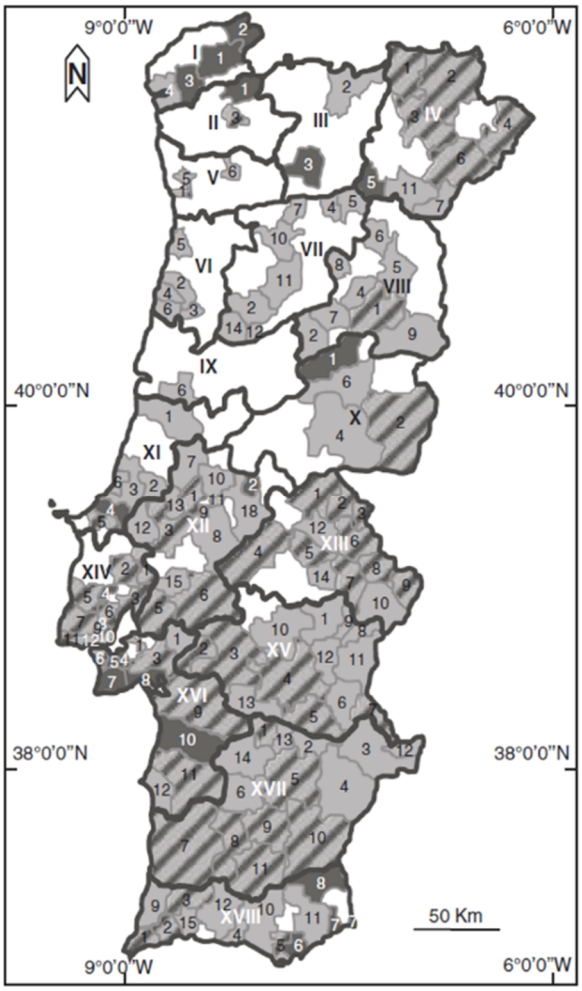

Fig.16 – Geographical distribution of *R. sanguineus* in Portugal by region and district, adapted from Santos-Silva et al (Santos-Silva et al., 2011). Grey areas symbolize old bibliographic records (before 1994). Dark areas correspond to new records produced by tick surveillance program or recent bibliographic references (after 1994). Striped dark and grey areas indicate data published in old literature and confirmed by Santos-Silva and colleagues (2011). Districts are numbered in roman numbers; "Concelhos" are presented as a painted area inside each district. Open marks represent bibliographic records without additional geographic details.

As mentioned above, 21 species of ixodids have been identified in Portugal (in detail in Table.3), several of which are recognized as vectors of PA that are able to infect human beings (Santos-Silva et al., 2006).

Table.3 - Ixodidae species present in Portugal, its' systematics and primary hosts, adapted from Santos-Silva et al. (2006).

Family Ixodidae				
Genus				
Rhipicephalus	***Ixodes***	***Dermacentor***	***Hyalomma***	***Haemaphysalis***
Species/Preferencial host				
R. sanguineus /Domestic Dogs	*I. ricinus* /Wild mammals	*D.marginatus* /Bovines	*H. lusitanicum* /Bovines	*Ha.punctata* /Small mammals
R. turanicus /Ovines	*I. hexagonus* /Wild mammals	*D.reticulatus* [2] /Dogs	*H.marginatum* /Bovines	*Ha.inermis* /Wild mammals
R. pusillus /Small wild mammals	*I. vespertillionis* /Chiropterans			*Ha.hispanica* /Small wild mammals
R. bursa /Caprines	*I. ventalloi*/ Small wild mammals			
R. annulatus [1] /Bovines	*I. bivari* /Small wild mammals			
	I. canisuga / Wild mammals			
	I. simplex /Chiropterans			
	I. acuminatus /Small wild mammals			
	I. frontalis /Wild birds			

[1] specie also denominated *Boophilus annulatus* ; [2] specie also denominated *Dermacentor pictus*

The *R. sanguineus* tick present one of the most widely host range among all tick species in Portugal, including domestic ruminants (bovine, ovine and caprine), wild animals (insectivores, non-human primates, carnivores, ungulates, lagomorphs, rodents, and birds), and domestic animals (dogs, cats and others) (Caeiro, 1999; Dias, 1994; Estrada-Peña and Santos-Silva, 2005; Santos-Silva et al., 2011). The domestic dog continues to have a prominent role as *R. sanguineus* main host in the country (Santos-Silva, 2010; Santos-Silva et al., 2011, 2006). This tick is also frequently collected from humans in Portugal, although past references reported that they feed rarely on humans (Parola et al., 2008; Santos-Silva et al., 2011; Walker et al., 2000).

Only three groups of genus *Rhipicephalus* exist in Portugal: *R. sanguineus* group (*R. sanguineus* and *R. pusillus*), *R. bursa* group, and the recently added to the genus *Rhipicephalus-Boophilus* group (*R. "Boophilus" annulatus*) (Guglielmone et al., 2010; Murrell and Barker, 2003; Santos-Silva et al., 2011). There were also three species of the *R. sanguineus* group were reported in the country, namely *R. sanguineus, R. turanicus* and *R. pusillus* (Caeiro, 1999; Dias, 1994; Estrada-Peña et al., 2004; Formosinho et al., 2006; Papadopoulos et al., 1992; Rosa et al., 2006; Rosalino et al., 2007; Santos-Silva et al., 2006; Tendeiro, 1962). However, some authors claim that the first two species populations in Portugal are genetically indistinguishable, characterized by a high level of morphological polymorphism (Santos-Silva, 2010).

The problem pointed is that this same morphological polymorphisms reported revealed the involvement of more than one species in *R. sanguineus s.l* species in European and

American *R. sanguineus* populations (Estrada-Peña and Sánchez, 1988; Oliveira et al., 2005; Papadopoulos et al., 1992; Ribeiro et al., 1996; Rosa et al., 2013; Tendeiro, 1962).

The most recent morphological and genetic study on this matter agreed with the fact that at least some populations of the *R. sanguineus s.l.* Portuguese populations are not *R. turanicus*, based on Filippova (1997) species' description, but instead make part of one lineage cluster until now not yet described: temperate species or southern lineage, cluster or group R. sp. type II (Dantas-Torres et al., 2013).

This observations emphasize the need of reevaluation of the intra and interspecific morphological characters between *R. sanguineus* and *R. turanicus* in Portuguese populations to clarify the actual presence of one or more species, or even of hybridization.

1.4.1. Associated Diseases in Portugal

One of the two most important species in terms of public health in Portugal is *R. sanguineus* (Santos-Silva et al., 2013), being responsible for the transmission to dogs of *B. canis vogeli*, *B. canis canis*, *E. canis*, *Ri. conorii*, *H.canis* (Alexandre et al., 2009; Cardoso et al., 2010; Santos-Silva, 2010; Santos-Silva et al., 2013) and a pathogen of unknown pathogenicity, *Ri. massiliae* (Santos-Silva *et al.*, 2013) in the country. Presumably, *Anaplasma platys* and *Leishmania infantum* may be also pathogens of this tick-vector in Portugal (Cardoso et al., 2010, 2008; Shaw et al., 2001).

All life stages of *R. sanguineus* can possibly infect humans, but the nymphs are the responsible for most of the Portuguese recorded cases in August and September, although the vector is active all year long in many regions of the country (Santos-Silva et al., 2013, 2011).

The Mediterranean spotted fever (MSF) is the most common tick-borne disease transmitted to humans in Portugal, and it is an endemic disease in the country (Bacellar et al., 1999; Oliveira and Côrte-Real, 1999; Santos-Silva et al., 2013). There are two strains responsible for MSF in the country: *R. conorii* Malish and *Ri. conorii israelensis* (or Israeli tick typhus) (Bacellar et al., 1999; Sousa et al., 2003a).

The MSF incidence rate are one of the highest among the Mediterranean basin countries, with an estimated incidence rate of $9.8/10^5$ inhabitants (the only data available being from

the period from 1989 to 2000), having the Alentejo region the highest rate reported ($31.4/10^5$ inhabitants), although Bragança is the district which appears with greater number of cases ($62/10^5$ inhabitants) (Louro et al., 2006; Marques et al., 2005; Oliveira and Côrte-Real, 1999; Santos-Silva et al., 2013; Sousa et al., 2003a). The mortality rate recorded in recent years in Portugal range between 2.3 and 23% (Abreu et al., 2007; Louro et al., 2006; Oliveira and Côrte-Real, 1999; Sousa et al., 2003b). Even though around 1000 MSF cases are reported in Portugal per year, many authors claim that this numbers are underrated, due to a suspected proportion of human-infections that pass unreported (Marques et al., 2005; Oliveira and Côrte-Real, 1999; Sousa et al., 2003a). It may easily happen in fact, because if some veterinarians treat sick animals without any laboratory diagnosis, it becomes impossible to quantify accurately the incidence and prevalence of PA tick-associated.

The most part of the reported cases of disease are registered in the summer time, mainly between July and August, due to the more favorable climatic conditions in that period to the arthropod-proliferation (Louro et al., 2006; Sousa et al., 2003a). But although the disease essentially occur in rural regions, there are many reports in urban and suburban areas nowadays, which is probably due to greater population mobility and increased contact and mobility of domestic animals, allowing for a greater vector (Louro et al., 2006).

Q fever is also a TBD transmitted by *R. sanguineus* endemic in Portugal, with an incidence of around 50 cases per year (Oliveira and Côrte-Real, 1999). The small number of cases associated with other species of rickettsia may be due to the fact that most of them do not have laboratory confirmation by molecular techniques, being identified only as rickettsia-disease, or even often mistaken for Boutonneuse fever (Santos-Silva et al., 2013).

It is then obvious that the subject is far from being closed, being mandatory to carry out more research in morphological, ultramorphological and genetic levels, as well as using more recent data on incidence of TBD across the country to detect with a higher certainty the presence of *R. sanguineus* Portuguese population ticks and its associated diseases.

2. THESIS CONTEXT AND OBJECTIVES

In spite of few studies have been carried out on this issue in Portugal, there is still much disagreement and controversy on the correct identification of *R. sanguineus* and *R. turanicus* in the country.

This work is then a preliminary morphological, ultramorphological and molecular study of three Portuguese populations of *R. sanguineus*, collected from dogs originated from Óbidos, Caldas da Rainha and Santarém. The reasons behind dogs as the source of specimens are multiple: they are the main host of this tick species, several TBD are of clinical importance in this animals (babesiosis and ehrliochiosis), and they live in close vicinity with human beings and can act as human infections reservoirs. The intent of this study is help to clarify and explain:

- The previous morphological variability identified in the populations of this species;
- The existence of more than one species or a polymorphic species of *R. sanguineus* in Portugal;
- The evaluation of the best morphological features to the correct identification of *R. sanguineus*;
- The quality of the COI molecular marker as a tool to identify and distinguish closely allied species of *Rhipicephalus spp.*;
- The identification of some morphological and genetic diversity in Portuguese specimens of *R. sanguineus*.

Questions to been answered:

i. Are *R. sanguineus* and *R. turanicus* Portuguese populations morphological and ultramorphologically distinguishable?

ii. Is the COI molecular marker suitable to determine that distinction?

iii. Are the chosen morphological features appropriate to distinguish the *R. sanguineus s.l.* species?

iv. Are the morphological approaches used useful to distinguish *R. sanguineus* and *R. turanicus* specimens?

3. MATERIALS AND METHODS

3.1. Ticks Collection

The ticks included in this study are part of the Zoological Collection of the Instituto de Investigação Científica Tropical (ZC/IICT, Lisboa, Portugal), and were obtained in dogs hosts between 2005 and 2012 in the districts of Óbidos, Caldas da Rainha, and Santarém. These places are known endemic areas of *R. sanguineus* ticks in Portugal. The specimens were all conserved in Alcohol 70%.

3.2. Morphological Study

For the morphological study, 426 representative ticks (137 females, and 289 males) were selected from a collection consisting of more than 3000 *R. sanguineus*-like specimens. Due to the fact that species identification by morphological criteria can be difficult, especially when specimens are physically damaged, engorged or in sub-adult stages, only adults were used as our study subjects. In the case of females, non-engorged or slightly engorged specimens were chosen. For a preliminary analysis of the data base, traditional taxonomic analysis of specimens was performed (identification to the species level) was performed, based on conventional keys and descriptions – *R. sanguineus* Travassos Dias (1994) and Walker (2003) descriptions, *R. turanicus* Papadopoulos (1992) and Walker (2003) descriptions; and the *R. pusillus* Travassos Dias (1994) descriptions.

Morphological features of the selected ticks were examined, measured or nominally classified (depending on whether they were quantifiable or qualifiable variables), and photographed using a light stereomicroscope (LS) associated to live measurement software LAS (Leica Application System, 2009), namely: scutum and conscutum length, width, punctation distribution and punctation size; conscutum width measured at post-paramedian grooves level; scutum posterior margin shape; cervical fields depression, shape and setiferous punctations presence; cervical grooves definition; basis capituli width and height; porose areas width, height, and distance between them; ventral-measured palps height; second palp shape; lateral grooves beginning and texture; posteromedian grooves length and deepness; paramedian grooves deepness and shape; parma presence; festoons count; adanal plates height, width, posterior margin shape, total

shape, and ending; spiracular areas maximum length, maximum width, thirds width, width of the tail beginning, tail ending width, angle relatively to the tail, and width of the adjacent festoon.

More than 4000 photos were taken. Examples of how these measures were taken can be consulted in Fig.17 and in Fig.18. 125 females' genital apertures were extracted from the specimens and mounted in a slide with Tendeiro's Liquid to clarify the internal structures. Around 70 photos were taken of this feature. Species-type groups were then formed, based on the morphological variability observed. From these groups, some representative subjects were selected for SEM analysis and others for molecular analysis. Light microscopy and SEM photos were then subjected to another taxonomic analysis, but this time based on Dantas-Torres et al. (2013) *R. sanguineus* group classification. All specimens that did not fit in these descriptions were grouped according to morphological similarities and renamed. This analysis was performed to the results that could be compared with the ones previously obtained.

Additionally, some of the other morphological features, described in the taxonomic-keys references indicated before, were taken into account to evaluate the best taxonomic cluster suitable to the specimen evaluated.

For better viewing, both figures (Fig.17 and Fig.18) are shown in the next page.

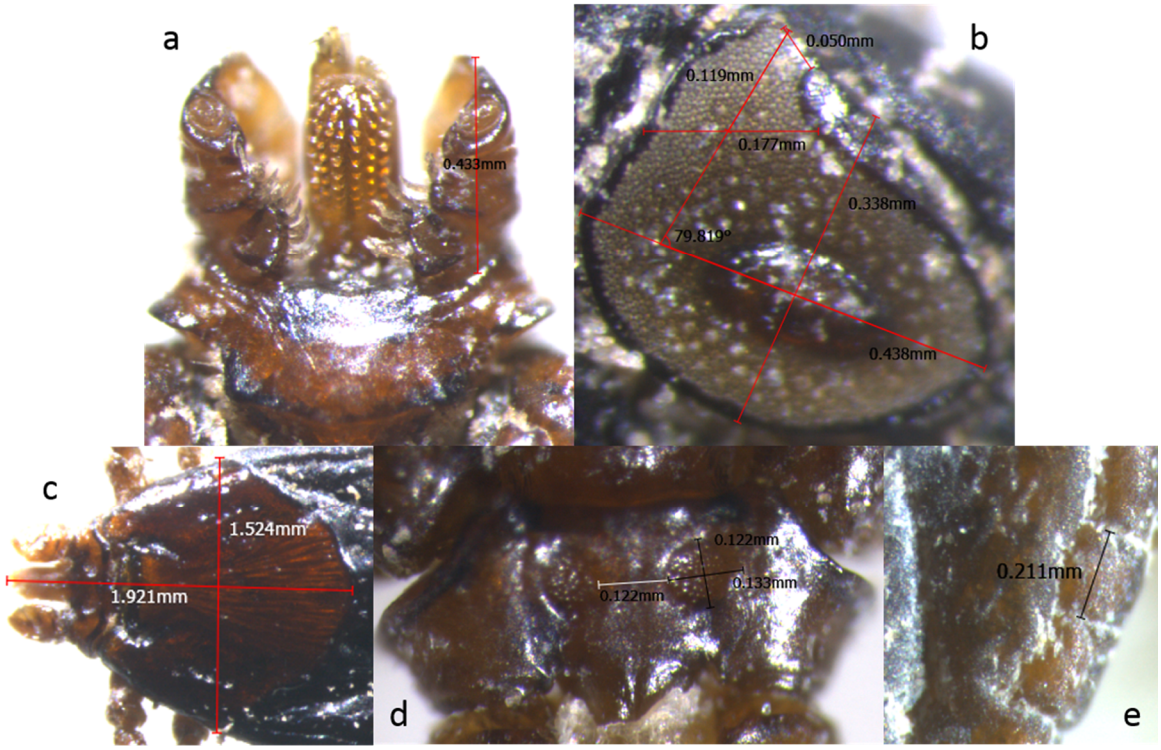

Fig.17 – Some measures taken using a light stereomicroscope associated to live measurement software LAS (Leica Application System, 2009). a) Ventral-measured palp height, from the base of the article 1; b) spiracular area beginning width of the tail (0.177mm), tail ending width (0.050mm), and angle relatively to the tail (79.819º); c) conscutum length (1.921mm) and width (1.524mm); d) porose areas width (0.133mm), height (0.122mm), and distance (0.122mm); e) width of the adjacent festoon.

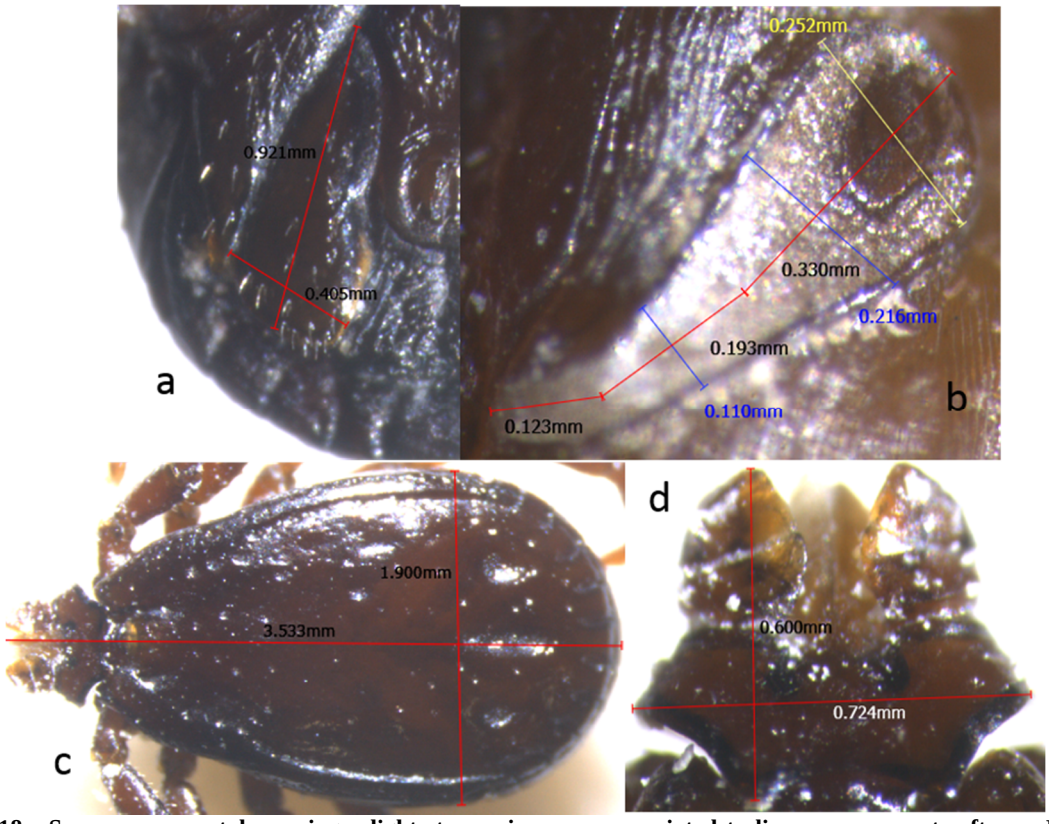

Fig.18 - Some measures taken using a light stereomicroscope associated to live measurement software LAS (Leica Application System, 2009). a) adanal plate height (0.921mm) and width (0.405mm), b) spiracular areas maximum length ((0.123+0.193+0.330)mm), maximum width (0.252mm), thirds widths (0.110 and 0.216mm), c) scutum length (3.533mm) and width (1.900mm), d) basis capituli width (0.724mm) and height (0.600mm).

3.3. Ultramorphologic Study

32 selected specimens were photographed using SEM (12 females and 30 males). They were chosen as representatives of the taxonomic groups formed in the morphological study. Additionally, 9 specimens (5 males and 4 females), that were not part of the study group but which are from Santarem district, were photograph as additional data. 283 SEM photos were taken by Telmo Nunes (Laboratory Technician, Microscopy Laboratory, Faculty of Sciences, University of Lisbon).

For this study, the material was dried for 16 hours, then fixed to a metal stand with double-faced adhesive tape, and finally sputtered with gold (1 hour at least). All morphology features referred in the morphological study were photographed and taxonomically classified.

3.4. Statistical Data Analyses

For the characterization and interpretation of the different variables considered in the morphological study, SPSS Statistics software (IBM Corporation) was used. Hierarchical Cluster Analysis (HCA) tool was applied, to quantitative and qualitative variables, aiming to obtain clusters of similar individuals. Cross-tabulation and the analysis of variance (ANOVA) statistics were performed for clusters characterization. For comparison purposes, the clusters obtained from the different variables analysis, and also the results of the traditional taxonomic analysis based on the morphological studied specimens, were subjected to Correspondence Analysis (CA). This analysis reduces the large dataset associations and thus provides for a better interpretation. Both female and male data were processed in two different analysis due to the morphological dimorphism presented.

3.5. Molecular Study

After the morphological study, 200 representative tick specimens were selected for molecular analysis. This analysis were applied in order to try to identify *Rhipicephalus spp.* morphological groups, and to characterize the used molecular marker' specificity. To achieve this, mDNA COI marker was used (Cruickshank, 2002; Folmer et al., 1994; Navajas et al., 1998). It was known from the literature that this marker was not very

specific to our analysis, but due to financial constraints, it was the available one in our laboratory, and we had the logistical conditions to work with it as well.

Experimental molecular procedures followed current methodologies (Appendix I, pg.126), starting with DNA extraction from parasite samples using a commercial E.Z.N.A.® Insect DNA Kit. DNA fragments under study were then amplified by PCR with specific primers (Appendix I, pg.126), and correct amplification was checked through electrophoresis. PCR products were then purified with the SureClean commercial kit (Bioline) and then sent to be sequenced in a specialized company (Macrogen Europe). Sequences obtained were then analysed by Sequencher and BioEdit, and the obtained data were compared with available sequences on GenBank online database (BLAST tool).

4. RESULTS

In short, we have selected 426 specimens for the preliminary morphological study, and based on traditional taxonomic analysis, 4 groups were formed. From these groups, 32 representative subjects were selected for SEM analysis and 200 representative specimens were selected for molecular analysis using the COI marker. Quantitative and qualitative variables associated to the morphological features referred were evaluated from the LS photographed specimens and subjected to statistical analysis. The LS and SEM specimens-photos were then subjected to taxonomic analysis based on the Dantas-Torres latter classification (2013) latter classification.

All the results were then compared.

4.1. Preliminary Morphological Classification

Morphological analysis based on the traditional morphological analysis (Dias, 1994; Papadopoulos et al., 1992; Walker et al., 2000; Walker et al., 2003) of the described features resulted in the following groups for both females and males specimens: "*R. sanguineus"*, "Intermediate*"*, "*R. turanicus"*, and "*R. pusillus"*. As a matter of statistical analysis convenience to results comparison, these groups were numbered from 1 to 4, respectively. The group "*R. pusillus"* is the control group.

The detailed results are present in Table.4 and Table.5. Descriptive statistics of the formed groups are shown in Table.6 and Table.7.

Table.4 – Females taxonomic groups formed by the traditional taxonomic classification, clusters formed by hierarchical statistical analysis in SPSS software (IBM Corporation) using the quantitative and qualitative variables, and their associated specimen identification number (ID) of the zoological IICT collection.

Disctrict	ID	Taxonomic Group	Quantitative Cluster	Qualitative Cluster	Disctrict	ID	Taxonomic Group	Quantitative Cluster	Qualitative Cluster
O	105	*R.sanguineus*	1	1	S	1061	*R. turanicus*	4	2
O	108	*R.sanguineus*	2	1	S	1064	*R. turanicus*	4	4
O	109	*R.sanguineus*	2	1	S	1065	*R. turanicus*	4	4
O	110	*R.sanguineus*	3	1	S	1066	*R. sanguineus*	3	2
O	111	*R.sanguineus*	1	1	S	1067	*R. turanicus*	3	4
O	119	*R.sanguineus*	4	1	S	1068	*R. sanguineus*	4	1
O	125	*R.sanguineus*	4	1	S	1069	*R. turanicus*	1	2
O	139	*R. pusillus*	3	2	S	1071	*R. sanguineus*	4	2
O	141	*R. pusillus*	2	1	S	1073	*R. turanicus*	4	4
O	143	*R. pusillus*	3	1	S	1090	*R. sanguineus*	4	2
O	160	*R.sanguineus*	1	1	S	1121	*R. sanguineus*	1	4
O	161	*R.sanguineus*	4	3	S	1122	*R. sanguineus*	4	4
O	173	*R.sanguineus*	3	1	S	1159	*R. pusillus*	1	3
O	179	*R.sanguineus*	1	3	S	1160	*R. turanicus*	4	1
O	192	*R.sanguineus*	4	3	S	1196	*R. sanguineus*	1	4
O	208	*R. turanicus*	4	1	S	1197	*R. sanguineus*	4	4
O	215	*R. sanguineus*	1	4	S	1198	*R. sanguineus*	4	1
O	216	*R. sanguineus*	1	1	S	1199	*R. turanicus*	3	4
O	241	*R. sanguineus*	1	4	S	1200	*Intermediate*	4	4
O	259	*R. sanguineus*	1	4	S	1206	*R. turanicus*	1	2
O	260	*R. sanguineus*	1	1	S	1214	*R. turanicus*	4	2
O	1340	*R. sanguineus*	1	1	S	1215	*R. turanicus*	1	1
O	1345	*R. sanguineus*	1	2	S	1216	*R. turanicus*	3	1
O	1346	*R. sanguineus*	1	1	S	1261	*R. turanicus*	1	2
O	1347	*R. sanguineus*	4	1	S	1262	*R. turanicus*	4	3
S	328	*R. sanguineus*	3	2	S	1263	*R. turanicus*	3	4
S	332	*R. sanguineus*	1	2	S	1264	*R. sanguineus*	1	2
S	339	*R. turanicus*	4	4	S	1267	*R. turanicus*	3	2
S	397	*R. sanguineus*	4	1	S	1269	*R. sanguineus*	1	2
S	435	*R. turanicus*	3	4	S	1271	*R. turanicus*	1	3
S	436	*R. sanguineus*	3	2	S	1272	*R. sanguineus*	1	4
S	465	*R. sanguineus*	4	1	S	1274	*R. sanguineus*	3	2
S	466	*R. sanguineus*	4	3	S	1275	*Intermediate*	3	3
S	522	*R. sanguineus*	3	4	S	1276	*Intermediate*	3	1
S	554	*R. turanicus*	1	1	S	1277	*R. sanguineus*	3	2
S	555	*R. sanguineus*	1	2	S	1282	*Intermediate*	4	4
S	562	*R. turanicus*	1	1	S	1283	*R. sanguineus*	1	4
S	563	*R. sanguineus*	1	4	S	1286	*R. sanguineus*	1	1
S	569	*R. sanguineus*	1	2	S	1288	*R. sanguineus*	1	1
S	575	*R. sanguineus*	4	3	S	1293	*R. sanguineus*	4	1
S	580	*R. turanicus*	3	4	S	1296	*R. sanguineus*	1	4
S	582	*Intermediate*	1	4	S	1298	*Intermediate*	1	4
S	589	*R. sanguineus*	1	2	S	1308	*R. sanguineus*	4	4
S	621	*R. turanicus*	4	4	S	1309	*R. sanguineus*	4	4
S	634	*R. turanicus*	3	1	S	1333	*R. pusillus*	3	3
S	708	*R. turanicus*	3	4	S	1334	*R. pusillus*	3	3
S	712	*R. sanguineus*	4	2	S	1485	*R. sanguineus*	3	3
S	827	*R. sanguineus*	3	1	S	1526	*R. sanguineus*	1	2
S	840	*R. sanguineus*	4	4	CR	1528	*R. turanicus*	3	1
S	845	*R. sanguineus*	4	4	CR	1529	*R. turanicus*	3	1
S	848	*R. sanguineus*	1	2	CR	1530	*R. turanicus*	3	4
S	853	*R. turanicus*	4	1	CR	1532	*R. turanicus*	3	4
S	859	*R. sanguineus*	4	2	CR	1533	*R. pusillus*	3	3
S	864	*R. sanguineus*	4	1	CR	1536	*R. turanicus*	1	4
S	943	*R. turanicus*	4	4	CR	1538	*R. turanicus*	3	4
S	944	*Intermediate*	4	2	CR	1544	*R. turanicus*	1	1
S	947	*R. sanguineus*	4	2	CR	1545	*R. turanicus*	3	4
S	948	*R. sanguineus*	4	2	CR	1551	*R. turanicus*	1	2
S	950	*Intermediate*	4	2	CR	1552	*R. sanguineus*	1	2
S	998	*R. sanguineus*	4	4	CR	1554	*R. turanicus*	3	1
S	1043	*R. sanguineus*	4	3	CR	1555	*R. turanicus*	4	3
S	1044	*R. turanicus*	3	2	CR	1558	*R. turanicus*	3	1
S	1046	*R. sanguineus*	1	4	CR	1559	*R. turanicus*	3	1
S	1052	*R. sanguineus*	4	4	CR	1560	*R. turanicus*	3	3
S	1053	*R. turanicus*	3	4	CR	1563	*R. turanicus*	4	2
S	1055	*R. sanguineus*	4	2	CR	1564	*R. turanicus*	3	2
S	1057	*R. sanguineus*	3	2	CR	1568	*R. pusillus*	1	3
S	1058	*R. sanguineus*	4	3	CR	1569	*R. pusillus*	1	3
S	1060	*R. turanicus*	4	4					

Table.5 - Males taxonomic groups formed by the traditional taxonomic classification, clusters formed by hierarchical statistical analysis in SPSS software (IBM Corporation) using the quantitative and qualitative variables, and their associated specimen identification number (ID) of the zoological IICT collection.

Disctrict	ID	Taxonomic Group	Quantitative Cluster	Qualitative Cluster	Disctrict	ID	Taxonomic Group	Quantitative Cluster	Qualitative Cluster
O	103	*R. sanguineus*	1	1	S	316	*R. sanguineus*	2	3
O	106	*R. sanguineus*	2	2	S	319	*R. sanguineus*	1	1
O	107	*R. sanguineus*	1	2	S	320	*Intermediate*	3	3
O	112	*R. sanguineus*	1	2	S	321	*Intermediate*	2	3
O	113	*R. sanguineus*	1	1	S	323	*R. sanguineus*	2	3
O	114	*R. sanguineus*	3	1	S	325	*R. sanguineus*	4	3
O	115	*R. sanguineus*	2	3	S	326	*R. sanguineus*	2	2
O	116	*R. sanguineus*	1	1	S	330	*R. sanguineus*	4	3
O	117	*R. sanguineus*	1	1	S	331	*Intermediate*	4	3
O	118	*R. sanguineus*	2	1	S	333	*R. turanicus*	3	3
O	123	*R. sanguineus*	4	2	S	336	*R. turanicus*	3	3
O	129	*R. turanicus*	3	3	S	337	*R. turanicus*	3	1
O	136	*R. sanguineus*	1	2	S	344	*R. turanicus*	3	3
O	137	*R. pusillus*	3	1	S	350	*Intermediate*	2	3
O	138	*R. sanguineus*	1	1	S	358	*R. turanicus*	3	1
S	142	*R. pusillus*	2	1	S	359	*R. turanicus*	3	2
S	145	*R. pusillus*	2	1	S	369	*R. turanicus*	3	4
O	155	*Intermediate*	3	1	S	371	*R. sanguineus*	2	3
O	156	*R. sanguineus*	2	2	S	374	*Intermediate*	3	2
O	157	*R. pusillus*	2	2	S	388	*R. sanguineus*	2	1
O	158	*R. sanguineus*	1	1	S	390	*R. sanguineus*	1	1
O	159	*R. sanguineus*	2	4	S	395	*R. sanguineus*	1	1
O	162	*R. sanguineus*	1	3	S	429	*R. sanguineus*	2	3
O	166	*R. sanguineus*	2	3	S	449	*R. turanicus*	3	1
O	167	*R. sanguineus*	2	3	S	452	*R. sanguineus*	2	3
O	168	*R. sanguineus*	1	1	S	453	*R. sanguineus*	2	3
O	169	*R. turanicus*	3	3	S	464	*Intermediate*	4	3
O	170	*R. turanicus*	1	1	S	475	*R. turanicus*	4	4
O	171	*R. sanguineus*	2	3	S	477	*R. sanguineus*	4	4
O	174	*R. sanguineus*	1	1	S	478	*R. sanguineus*	2	1
O	175	*R. sanguineus*	3	1	S	491	*R. sanguineus*	2	1
O	177	*R. sanguineus*	2	4	S	531	*R. sanguineus*	4	3
O	180	*R. sanguineus*	2	3	S	572	*Intermediate*	2	2
O	181	*R. sanguineus*	2	3	S	583	*R. sanguineus*	2	2
O	182	*R. sanguineus*	1	1	S	597	*R. sanguineus*	4	3
O	183	*R. sanguineus*	3	1	S	609	*R. sanguineus*	2	2
O	184	*R. sanguineus*	1	1	S	613	*R. sanguineus*	4	3
O	185	*R. sanguineus*	1	1	S	630	*R. turanicus*	3	4
O	189	*R. sanguineus*	2	2	S	637	*R. sanguineus*	2	3
O	190	*R. sanguineus*	1	3	S	641	*R. sanguineus*	4	3
O	191	*R. sanguineus*	2	3	S	643	*R. sanguineus*	2	3
O	193	*R. sanguineus*	2	4	S	676	*R. sanguineus*	4	4
O	196	*R. sanguineus*	1	1	S	680	*R. sanguineus*	4	3
O	197	*R. sanguineus*	4	1	S	709	*R. sanguineus*	2	3
O	198	*R. sanguineus*	1	1	S	710	*R. turanicus*	1	2
O	199	*R. sanguineus*	2	3	S	711	*R. sanguineus*	4	1
O	200	*R. sanguineus*	2	4	S	714	*R. sanguineus*	4	3
O	202	*R. sanguineus*	2	2	S	716	*R. sanguineus*	1	2
O	211	*R. sanguineus*	4	3	S	717	*R. sanguineus*	1	2
O	212	*R. sanguineus*	2	4	S	738	*R. sanguineus*	2	1
O	224	*R. sanguineus*	1	3	S	824	*R. sanguineus*	4	3
O	234	*R. sanguineus*	2	3	S	829	*Intermediate*	4	3
O	235	*R. sanguineus*	1	4	S	861	*R. sanguineus*	2	3
O	236	*R. sanguineus*	2	4	S	890	*Intermediate*	2	3
O	237	*R. sanguineus*	2	4	S	900	*R. sanguineus*	1	1
O	239	*R. sanguineus*	2	3	S	945	*R. turanicus*	3	1
O	261	*R. sanguineus*	2	1	S	967	*R. sanguineus*	2	3
O	262	*R. sanguineus*	2	3	S	968	*R. sanguineus*	2	4
O	263	*R. sanguineus*	4	3	S	1047	*Intermediate*	1	2
O	227	*R. sanguineus*	2	3	S	1050	*Intermediate*	1	2
S	273	*Intermediate*	4	1	S	1076	*R. sanguineus*	1	1
S	276	*R. sanguineus*	3	1	S	1077	*R. sanguineus*	1	2
S	284	*R. sanguineus*	4	2	S	1079	*R. sanguineus*	1	1
S	285	*R. sanguineus*	4	3	S	1081	*R. sanguineus*	2	3
S	295	*R. sanguineus*	2	1	S	1082	*R. sanguineus*	2	1
S	296	*R. sanguineus*	2	2	S	1083	*Intermediate*	1	2
S	297	*R. sanguineus*	4	1	S	1084	*Intermediate*	1	2
S	298	*Intermediate*	2	3	S	1087	*Intermediate*	2	3
S	299	*R. sanguineus*	4	2	S	1103	*R. sanguineus*	2	3
S	302	*R. sanguineus*	4	3	S	1104	*R. sanguineus*	1	2
S	303	*R. sanguineus*	4	2	S	1105	*R. sanguineus*	1	3
S	306	*R. sanguineus*	4	2	S	1106	*R. sanguineus*	2	3

(Table.5 continued)

Disctrict	ID	Taxonomic Group	Quantitative Cluster	Qualitative Cluster	Disctrict	ID	Taxonomic Group	Quantitative Cluster	Qualitative Cluster
S	1107	*R. sanguineus*	2	1	S	1230	*Intermediate*	4	3
S	1108	*R. sanguineus*	4	3	S	1231	*R. sanguineus*	2	3
S	1109	*Intermediate*	4	4	S	1232	*R. sanguineus*	2	1
S	1110	*R. sanguineus*	4	4	S	1233	*R. sanguineus*	4	3
S	1111	*R. sanguineus*	1	1	S	1234	*R. sanguineus*	2	1
S	1112	*R. sanguineus*	2	3	S	1235	*R. sanguineus*	4	1
S	1113	*R. sanguineus*	2	3	S	1237	*Intermediate*	1	2
S	1114	*R. sanguineus*	2	3	S	1238	*R. sanguineus*	4	3
S	1115	*R. sanguineus*	1	1	S	1239	*R. sanguineus*	2	3
S	1125	*R. sanguineus*	1	1	S	1240	*R. sanguineus*	2	2
S	1126	*R. sanguineus*	1	1	S	1241	*R. sanguineus*	4	4
S	1127	*R. sanguineus*	1	1	S	1243	*R. sanguineus*	4	3
S	1128	*R. sanguineus*	2	4	S	1244	*R. sanguineus*	1	1
S	1129	*R. sanguineus*	1	1	S	1245	*R. sanguineus*	1	1
S	1130	*R. turanicus*	3	4	S	1246	*R. sanguineus*	4	3
S	1137	*R. sanguineus*	2	3	S	1247	*R. sanguineus*	4	3
S	1151	*R. sanguineus*	1	2	S	1248	*R. sanguineus*	1	3
S	1152	*R. sanguineus*	4	3	S	1249	*R. sanguineus*	2	4
S	1153	*R. sanguineus*	2	4	S	1250	*R. sanguineus*	4	3
S	1154	*R. sanguineus*	4	4	S	1251	*R. sanguineus*	4	3
S	1155	*R. sanguineus*	4	3	S	1252	*R. sanguineus*	2	2
S	1156	*R. sanguineus*	1	2	S	1253	*R. sanguineus*	4	3
S	1161	*R. sanguineus*	1	3	S	1254	*R. sanguineus*	4	3
S	1162	*R. sanguineus*	2	3	S	1255	*R. sanguineus*	4	3
S	1163	*R. sanguineus*	1	3	S	1256	*R. sanguineus*	2	3
S	1164	*R. sanguineus*	2	3	S	1257	*R. sanguineus*	4	1
S	1165	*R. sanguineus*	2	3	S	1258	*R. sanguineus*	1	1
S	1166	*R. sanguineus*	2	3	S	1259	*R. sanguineus*	4	3
S	1167	*R. sanguineus*	2	4	S	1260	*R. sanguineus*	4	1
S	1168	*Intermediate*	2	3	S	1323	*R. sanguineus*	2	3
S	1169	*R. sanguineus*	2	4	S	1324	*R. sanguineus*	2	3
S	1170	*R. sanguineus*	2	3	S	1325	*R. sanguineus*	2	3
S	1171	*R. sanguineus*	2	2	S	1326	*Intermediate*	2	2
S	1172	*Intermediate*	2	3	S	1327	*R. sanguineus*	4	3
S	1174	*R. sanguineus*	2	3	S	1328	*R. sanguineus*	2	3
S	1175	*Intermediate*	2	4	S	1329	*R. sanguineus*	4	2
S	1176	*R. sanguineus*	2	3	S	1476	*R. sanguineus*	2	3
S	1177	*R. sanguineus*	4	3	S	1481	*Intermediate*	4	4
S	1178	*R. sanguineus*	4	3	S	1488	*R. sanguineus*	2	2
S	1179	*R. sanguineus*	4	3	S	1489	*R. sanguineus*	2	3
S	1180	*R. sanguineus*	2	4	S	1525	*R. turanicus*	2	1
S	1181	*R. sanguineus*	2	3	CR	1527	*R. turanicus*	3	1
S	1182	*R. sanguineus*	4	3	CR	1531	*R. pusillus*	3	2
S	1183	*Intermediate*	2	3	CR	1534	*R. sanguineus*	2	1
S	1184	*R. sanguineus*	2	3	CR	1535	*R. sanguineus*	2	4
S	1186	*R. sanguineus*	2	4	CR	1537	*R. turanicus*	3	1
S	1187	*Intermediate*	2	4	CR	1539	*R. turanicus*	3	3
S	1188	*Intermediate*	2	2	CR	1540	*R. turanicus*	2	3
S	1189	*Intermediate*	3	3	CR	1541	*R. turanicus*	2	3
S	1190	*R. sanguineus*	1	3	CR	1542	*R. turanicus*	3	1
S	1191	*R. sanguineus*	2	3	CR	1543	*R. turanicus*	3	2
S	1192	*R. sanguineus*	4	4	CR	1546	*R. sanguineus*	2	1
S	1194	*R. sanguineus*	4	3	CR	1547	*Intermediate*	2	3
S	1195	*R. sanguineus*	4	1	CR	1548	*R. sanguineus*	2	2
S	1205	*Intermediate*	4	3	CR	1549	*R. sanguineus*	2	4
S	1207	*R. sanguineus*	4	1	CR	1550	*R. sanguineus*	2	4
S	1208	*R. sanguineus*	1	2	CR	1553	*R. sanguineus*	3	1
S	1209	*R. sanguineus*	2	3	CR	1556	*R. sanguineus*	2	3
S	1210	*R. sanguineus*	4	3	CR	1557	*Intermediate*	3	2
S	1211	*R. sanguineus*	4	2	CR	1561	*Intermediate*	2	2
S	1212	*R. sanguineus*	4	3	CR	1562	*R. sanguineus*	4	3
S	1213	*R. sanguineus*	1	1	CR	1565	*R. sanguineus*	2	2
S	1219	*Intermediate*	1	1	CR	1566	*R. sanguineus*	2	2
S	1220	*Intermediate*	4	3	CR	1567	*R. sanguineus*	2	1
S	1221	*R. sanguineus*	2	3	CR	1570	*Intermediate*	2	3
S	1222	*R. sanguineus*	4	1	CR	1571	*Intermediate*	1	2
S	1223	*R. sanguineus*	1	2	CR	1572	*R. sanguineus*	2	4
S	1224	*R. sanguineus*	2	3	CR	1573	*Intermediate*	2	2
S	1225	*R. sanguineus*	2	4	CR	1574	*R. sanguineus*	4	1
S	1226	*R. sanguineus*	1	1	CR	1575	*R. turanicus*	3	1
S	1227	*R. sanguineus*	4	4	CR	1576	*R. sanguineus*	2	1
S	1228	*R. sanguineus*	4	4	CR	1577	*R. sanguineus*	4	4

Table.6 – Females taxonomic groups, formed by traditional taxonomic classification, descriptive statistics for quantitative variable (morphologic feature). All measures were taken in millimeters, less the angle, taken in angle degrees. N- number of elements within the clusters, Std. Deviation - standard deviation.

Morphological Feature	Taxonomic Group	Descriptive Measures				
		N	Mean	Std. Deviation	Minimum	Maximum
Porose areas distance	*R.sanguineus*	73	0,111	0,019	0,020	0,153
	Intermidiate	8	0,115	0,009	0,094	0,121
	R.turanicus	47	0,116	0,018	0,072	0,161
	R.pusillus	9	0,101	0,012	0,080	0,122
	Total	137	0,112	0,018	0,020	0,161
Ventral-measured Palp height	*R.sanguineus*	73	0,442	0,031	0,365	0,505
	Intermidiate	8	0,447	0,029	0,409	0,492
	R.turanicus	47	0,434	0,038	0,350	0,508
	R.pusillus	9	0,377	0,070	0,290	0,481
	Total	137	0,435	0,040	0,290	0,508
Spiracular angle	*R.sanguineus*	73	86,964	11,191	63,261	126,048
	Intermidiate	8	88,349	8,399	76,697	97,644
	R.turanicus	47	80,607	11,124	53,010	101,316
	R.pusillus	9	82,320	13,371	61,136	96,328
	Total	137	84,559	11,488	53,010	126,048
Scutum lenght/width ratio	*R.sanguineus*	73	1,437	0,104	1,073	1,639
	Intermidiate	8	1,457	0,078	1,329	1,575
	R.turanicus	47	1,432	0,067	1,298	1,597
	R.pusillus	9	1,351	0,254	0,743	1,547
	Total	137	1,431	0,109	0,743	1,639
Basis Capituli lenght/width ratio	*R.sanguineus*	73	0,834	0,057	0,667	0,954
	Intermidiate	8	0,871	0,025	0,832	0,897
	R.turanicus	47	0,843	0,046	0,760	0,939
	R.pusillus	9	0,835	0,034	0,792	0,906
	Total	137	0,839	0,051	0,667	0,954
Porose areas height/width ratio	*R.sanguineus*	73	1,091	0,141	0,814	1,494
	Intermidiate	8	1,097	0,099	0,967	1,236
	R.turanicus	47	1,094	0,113	0,775	1,375
	R.pusillus	9	1,057	0,124	0,950	1,327
	Total	137	1,090	0,128	0,775	1,494
Spiracle Oval area height/width ratio	*R.sanguineus*	73	0,814	0,185	0,475	1,940
	Intermidiate	8	0,778	0,049	0,702	0,855
	R.turanicus	47	0,805	0,097	0,399	0,979
	R.pusillus	9	0,819	0,148	0,682	1,118
	Total	137	0,809	0,151	0,399	1,940
Spiracular tail length/widths-diference ratio	*R.sanguineus*	73	1,136	0,240	0,239	1,672
	Intermidiate	8	1,153	0,144	0,908	1,356
	R.turanicus	47	1,055	0,182	0,695	1,554
	R.pusillus	9	1,323	0,604	0,777	2,837
	Total	137	1,122	0,262	0,239	2,837

Table.7 - Males taxonomic groups, formed by traditional taxonomic classification, descriptive statistics for quantitative variable (morphologic feature). All measures were taken in millimeters, less the angle, taken in angle degrees. N- number of elements within the clusters, Std. Deviation - standard deviation.

Morphological Feature	Taxonomic Group	Descriptive Measures				
		N	Mean	Std. Deviation	Minimum	Maximum
Ventral-measured Palp height	*R. sanguineus*	221	0,370	0,036	0,247	0,515
	Intermediate	38	0,371	0,037	0,298	0,436
	R. turanicus	25	0,353	0,034	0,278	0,433
	R. pussilus	5	0,263	0,025	0,242	0,303
	Total	289	0,366	0,039	0,242	0,515
Spiracular angle	*R. sanguineus*	221	138,249	16,523	90,706	178,456
	Intermediate	38	138,822	17,695	77,345	173,660
	R. turanicus	25	139,775	15,484	98,053	177,198
	R. pussilus	5	138,777	10,907	127,448	154,648
	Total	289	138,465	16,450	77,345	178,456
Conscutum lenght/width ratio	*R. sanguineus*	221	1,743	0,139	1,470	3,214
	Intermediate	38	1,728	0,108	1,429	1,998
	R. turanicus	25	1,689	0,105	1,390	1,885
	R. pussilus	5	1,734	0,090	1,627	1,842
	Total	289	1,736	0,133	1,390	3,214
Basis Capituli lenght/width ratio	*R. sanguineus*	221	0,855	0,061	0,649	0,992
	Intermediate	38	0,848	0,067	0,688	0,961
	R. turanicus	25	0,872	0,068	0,673	0,964
	R. pussilus	5	0,845	0,077	0,775	0,950
	Total	289	0,855	0,062	0,649	0,992
Spiracle Oval area height/width ratio	*R. sanguineus*	221	2,438	0,273	1,708	3,768
	Intermediate	38	2,302	0,200	1,847	2,672
	R. turanicus	25	2,167	0,510	1,601	4,440
	R. pussilus	5	2,513	0,444	2,097	3,000
	Total	289	2,398	0,306	1,601	4,440
Spiracular area third-widths ratio	*R. sanguineus*	221	1,702	0,272	0,543	2,394
	Intermediate	38	1,644	0,256	1,213	2,229
	R. turanicus	25	1,431	0,224	1,040	1,919
	R. pussilus	5	1,381	0,286	0,976	1,782
	Total	289	1,665	0,278	0,543	2,394
Adanal Plates height/width ratio	*R. sanguineus*	221	2,231	0,270	0,371	3,237
	Intermediate	38	2,233	0,254	1,650	2,822
	R. turanicus	25	2,224	0,209	1,846	2,578
	R. pussilus	5	1,961	0,258	1,704	2,353
	Total	289	2,226	0,264	0,371	3,237
Spiracular tail final width /adjacent festoon width ratio	*R. sanguineus*	221	0,246	0,075	0,106	0,520
	Intermediate	38	0,308	0,143	0,135	0,837
	R. turanicus	25	0,469	0,144	0,236	0,754
	R. pussilus	5	0,412	0,192	0,257	0,628
	Total	289	0,276	0,116	0,106	0,837
After posteromedian grooves measured width/conscutum width ratio	*R. sanguineus*	221	0,897	0,089	0,695	1,684
	Intermediate	38	0,897	0,070	0,789	1,025
	R. turanicus	25	0,867	0,049	0,798	0,978
	R. pussilus	5	0,822	0,072	0,695	0,868
	Total	289	0,893	0,084	0,695	1,684

4.2. Statistical Analysis Results

4.2.1. Females Morphological Features Analysis

Of the 137 females analysed, 25 (18.2%) of them were collected in Óbidos, 92 (67.2%) in Santarém, and 20 (14.6%) in Caldas da Rainha districts. In this study, we are not going to take into account the geographical distribution of the specimens due to the low number of samples collected.

- **Traditional taxonomic analysis**

Traditional taxonomic analysis was performed, and four clusters were formed: (1) *R. sanguineus* cluster, (2) *Intermediate* cluster, (3) *R. turanicus* cluster, (4) *R. pusillus* cluster. *R. pusillus* specimens were included in the study as an outlier group for control purposes. *Intermediate* specimens are the ones that cannot be identified as *R. sanguineus* nor *R. turanicus*, because present morphological features described for both species.

Of the total female specimens analysed, 73 (53.3%) were identify as *R. sanguineus*, 8 (5.8%) as Intermediate, 47 (34.3%) as *R. turanicus*, and 9 (6.6%) as *R. pusillus*. These results are shown in Fig.19.

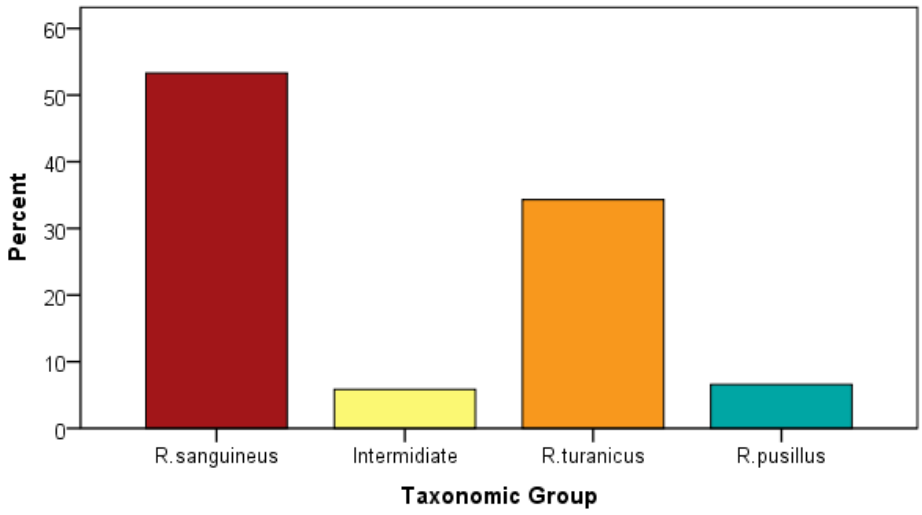

Fig.19 – Graphic representation of the female specimens' percentage per taxonomic group formed. Of the 137 female specimens analyzed, 73 (53.3%) were identify as *R. sanguineus*, 8 (5.8%) were identify as Intermediate, 47 (34.3%) were identify as *R. turanicus*, and 9 (6.6%) were identify as *R. pusillus*.

▪ Hierarchical Cluster Analysis

Hierarchical Cluster Analysis using SPSS was performed in separate for quantitative variables and for qualitative analysis, using the square of the Euclidian distance. The obtained dendrograms are in Fig.20 and Fig.21.

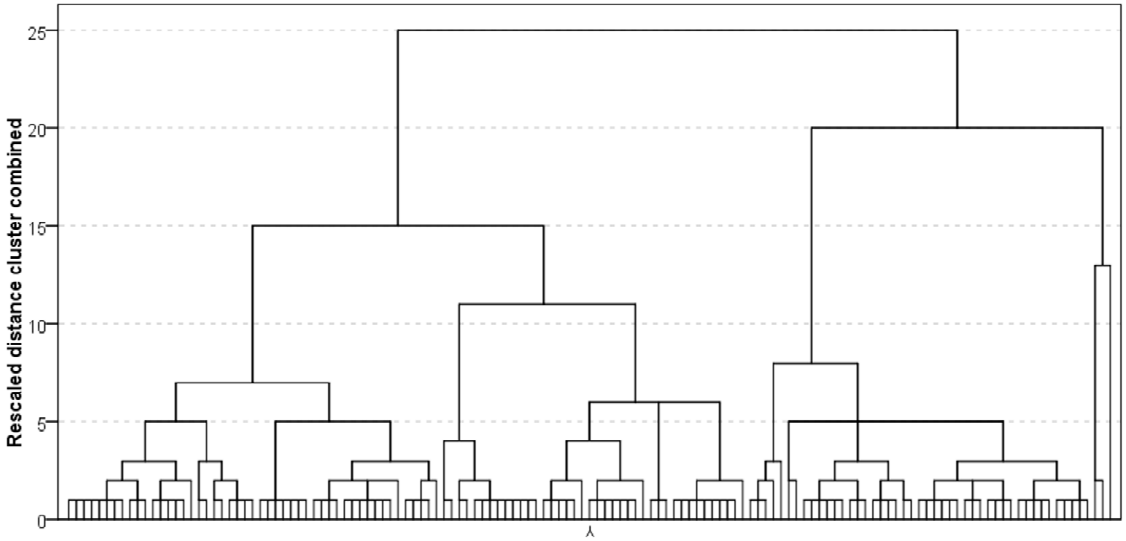

Fig.20 – Cluster Hierarchical Analysis dendrogram obtain with females' quantitative variables data. The higher distance between fusion coeficients were obtained in the rescaled distance value 14 (forming 4 clusters).

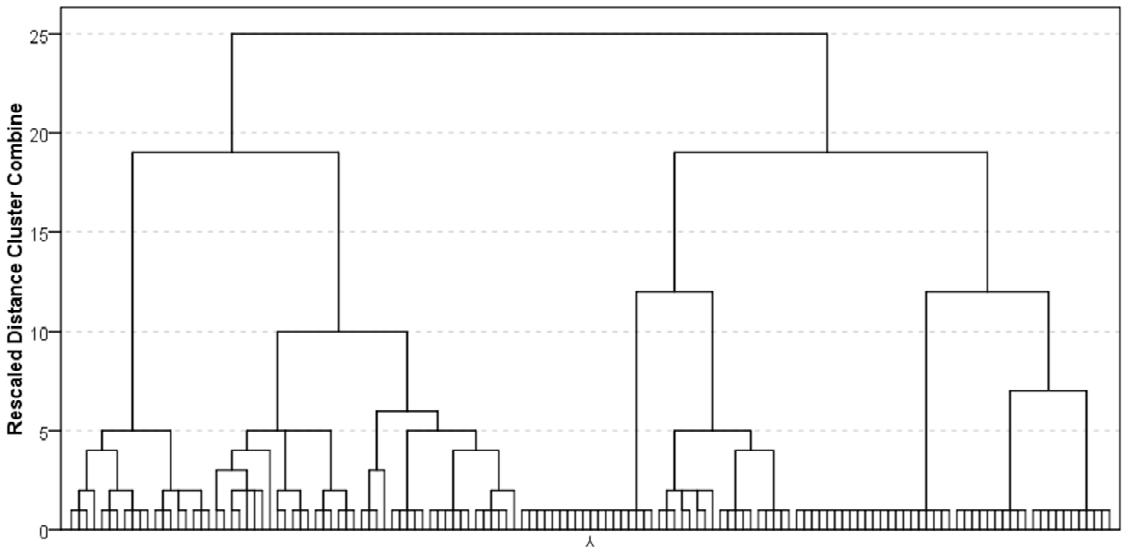

Fig.21 – Cluster Hierarchical Analysis dendrogram obtain with females' qualitative variables data. The higher distance between fusion coeficients were obtained in the rescaled distance value 15 (forming 4 clusters).

Based on the fusion coefficients distances obtained during the cluster analysis (used for the formation of clusters, see Appendix II, pg. 127, Table I.1), we chose four groups to be formed in both quantitative and qualitative variable analysis. This choice is in accordance with the data observed in the dendrograms in Fig.20 and Fig.21.

Thereafter, and with a cluster profile characterization purpose, ANOVA statistical model and cross-tabulation statistics was performed for quantitative and qualitative variables clusters, respectively.

- ***Quantitative Variables Clusters Analysis***

To classify the clusters formed based on females' quantitative variables, a one-way ANOVA statistical analysis was performed. The table referent to the descriptive measures of the variables within the clusters is presented in Table.8.

The mean was the descriptive statistic measure used to describe these clusters. In Fig.22 and Fig.23, are graphically shown the means of the quantitative variables within the 4 clusters formed. Because the 'Spiracular angle quantitative' variable show the highest unit values (measured in angle degrees), and consequently highest standard deviation between clusters in comparison to the others quantitative variables included on the study (which is easily explained by the fact that this variable is the only one not expressed in millimetres), it means graphic is apart of the others. For more detailed data, see Table.8 and Fig.22.

Table. 8– Females descriptive statistics of quantitative variables within the clusters formed by hierarchical cluster analysis. All measures were taken in millimeters, less the angle, taken in angle degrees. N- number of elements within the clusters, Std. Deviation - standard deviation.

Morphological feature	Cluster	Descriptives Measures				
		N	Mean	Std. Deviation	Minimum	Maximum
Porose areas distance	1	45	0,111	0,021	0,020	0,148
	2	3	0,121	0,017	0,110	0,141
	3	40	0,118	0,019	0,080	0,161
	4	49	0,108	0,015	0,072	0,142
	Total	137	0,112	0,019	0,020	0,161
Ventral-measured Palp height	1	45	0,437	0,031	0,365	0,503
	2	3	0,441	0,011	0,429	0,451
	3	40	0,403	0,043	0,290	0,473
	4	49	0,459	0,024	0,409	0,508
	Total	137	0,435	0,040	0,290	0,508
Spiracular angle	1	45	91,644	9,410	70,481	126,048
	2	3	100,375	16,531	88,605	119,275
	3	40	77,054	9,005	53,010	90,489
	4	49	83,211	10,200	63,261	101,433
	Total	137	84,559	11,488	53,010	126,048
Scutum lenght/width ratio	1	45	1,392	0,087	1,073	1,548
	2	3	1,207	0,403	0,743	1,471
	3	40	1,422	0,080	1,257	1,569
	4	49	1,487	0,082	1,252	1,639
	Total	137	1,431	0,109	0,743	1,639
Basis Capituli lenght/width ratio	1	45	0,796	0,045	0,667	0,877
	2	3	0,837	0,025	0,808	0,855
	3	40	0,850	0,044	0,760	0,954
	4	49	0,871	0,032	0,783	0,939
	Total	137	0,839	0,051	0,667	0,954
Porose areas height/width ratio	1	45	1,027	0,100	0,814	1,276
	2	3	1,080	0,101	0,983	1,184
	3	40	1,124	0,125	0,924	1,494
	4	49	1,121	0,135	0,775	1,386
	Total	137	1,090	0,128	0,775	1,494
Spiracular Oval area height/width ratio	1	45	0,788	0,089	0,475	0,982
	2	3	1,547	0,412	1,118	1,940
	3	40	0,824	0,072	0,682	1,007
	4	49	0,771	0,098	0,399	0,990
	Total	137	0,809	0,151	0,399	1,940
Spiracular tail length/widths-difference ratio	1	45	1,201	0,179	0,748	1,632
	2	3	1,841	0,875	1,201	2,837
	3	40	0,993	0,238	0,239	1,365
	4	49	1,109	0,187	0,816	1,672
	Total	137	1,122	0,262	0,239	2,837

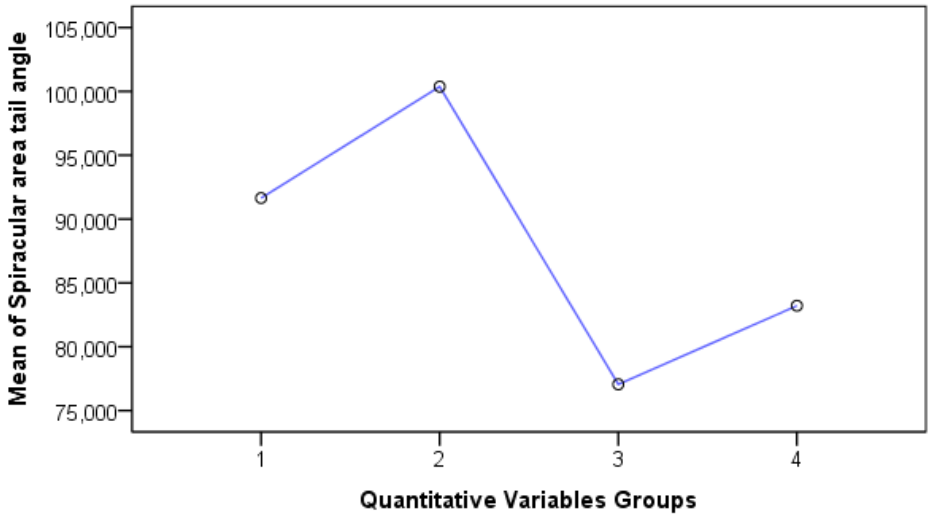

Fig.22 – 'Spiracular angle quantitative' variable females clusters means. This variable present the highest unit values (degree) and highest standard deviation in comparison to the other quantitative variables. All the clusters are well defined by its mean: Cluster 1 – μ=91.644, Cluster 2 – μ=100.375, Cluster 3 – μ=77.054, and Cluster4 – μ=83.211.

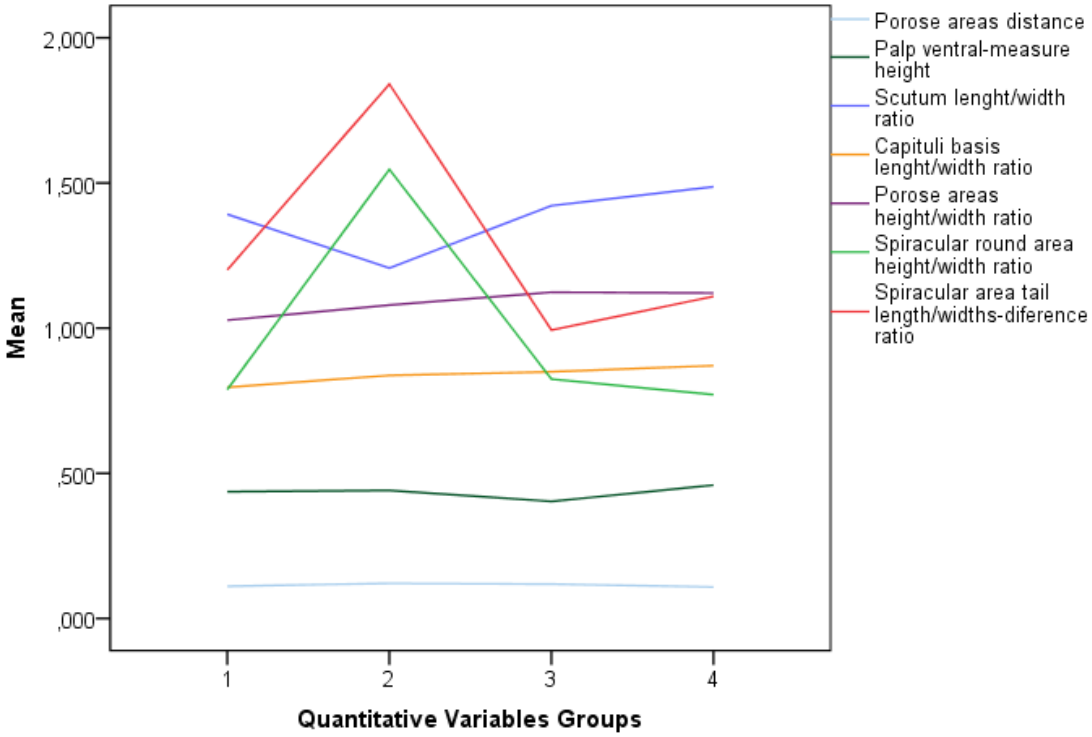

Fig.23 – Clusters means obtained based on all quantitative variables of females less the Spiracular angle. The "Spiracular tail length/widths-difference ratio" and the "Spiracle Oval area height/width ratio" variables are the ones where the mean clearly defines the different clusters, presenting the highest standard deviations by a descending order, immediately after the "Spiracular angle quantitative" variable. They are followed by the "Scutum length/width ratio" variable, with a lower standard deviation, but a still perceptible mean variation between the 4 clusters.

By evaluating the Table.8, Fig.22 and Fig.23, and in contribution descending order to the differentiation between clusters' means, we consider:

- The 'Spiracular angle' and 'Capituli basis length/width ratio' variables, which are the ones which means clearly defines the different clusters;
- The 'Spiracular tail length/widths-difference ratio', the 'Scutum length/width ratio', and the 'Ventral-measured palp height' variables, which just did not clearly differentiated the clusters with medium means (1-4, 1-3, and 1-2 clusters respectively), even so, a slightly difference is recognizable);
- The 'Porose areas height/width ratio', the 'Porose areas distance', and the 'Spiracle Oval area height/width ratio' variables did not differentiated at least one pair of cluster means (3-4, 1-4, and 1-3-4 clusters respectively), making of them the variables with the lowest contributes to the means clusters differentiation.

These results show that the cluster 1 has the ticks with the lowest length/width ratios for basis capituli; the cluster 2 has the ticks with the higher spiracular angles, the higher spiracular tail length/widths-difference ratio (biggest and largest spiracular areas), associated to the smallest scutums (length/width ratio); the cluster 3 has the ticks with the lowest spiracular angles, smallest palps heights, and lowest spiracular area length/widths ratio (smallest and thinner spiracles); and the cluster 4 present the biggest basis capituli (length/width ratio), biggest palps (in height) and biggest scutums (length/width ratio).

In order to evaluate if the clusters are significantly different, we proceeded with an ANOVA statistical analysis. The follow values were obtained:

- The 'Porose areas distance' variable result in F=2.564 and p=0.057, this is, the variable did not statistical significantly differentiated the between cluster' means.
- The 'Porose areas height/width ratio' variable result in F=6.118 and p=0.001, the 'Scutum length/width ratio' variable result in F=13.532 and p=0.000, the 'Spiracular tail length/widths-difference ratio' variable result in F=16.263 and p=0.000, the 'Spiracular angle' variable result in F=18.831 and p=0.000, the 'Ventral-measured palp height' variable result in F=21.503 and p=0.000, the 'Capituli basis length/width ratio' variable result in F=27.920 and p=0.000 and the 'Spiracle Oval area height/width ratio' variable result in F=56.484 and p=0.000. Therefore, all this variables statistical significantly

differentiated the cluster' means, that is, all of them contribute significantly for the clusters formation.

Based on the F-value, the variables are in crescendo order of significance for the cluster formation, this is, the 'Porose areas height/width ratio' variable were the one that less significantly contributed for the clusters 1 and 2 differentiation, and the 'Spiracle Oval area height/width ratio' variable were the one that gave the biggest contribute to the cluster 2 formation (the only one that this variable distinguished).

Moreover, the 'Scutum length/width ratio' differentiated mainly the 2, but also the 4; 'Spiracular tail length/widths-difference ratio' differentiated the cluster 2, as the 'Spiracle Oval area height/width ratio', which make this two variables consistent to each other (important because they express measures of the same morphological feature); 'Ventral-measured palp height' distinguished the 3 and 4 clusters; and the 'Spiracular angle' and the 'Capituli basis length/width ratio' distinguished all clusters, as referred before.

Relatively to the multiple comparison Tukey HSD test (post hoc test), which is a single-step multiple comparison procedure normally used in conjunction with ANOVA statistical tool to find means that are significantly different from each other, statistically significant p-values were observed. For note, and has the 'Porose areas distance' variable did not result in the exclusion of the H_0 hypothesis ($p>0.050$), it cannot be evaluated by this test.

'Scutum length/width ratio' variable just did not present a statistically significant difference between 1-3 clusters' means ($p=0.489$). 'Basis capituli length/width ratio' variable did not present a statistically significant difference between the 3 and the 4 clusters' means ($p=0.070$), and neither between the 2 and the rest of the clusters' means ($p=0.325$, 0.957, and 0.503 relatively to the clusters 1, 3 and 4 means). 'Spiracular tail length/widths-difference ratio' variable did not present a statistically significant difference between the 1-4 and 3-4 clusters' means ($p=0.209$ and 0.081 respectively). 'Ventral-measured palp height' variable did not present a statistically significant difference between the 2 and the rest of the clusters' means ($p=0.997$, 0.226 and 0.782 relatively to the clusters 1, 3 and 4 means). 'Spiracular angle' variable did not present a statistically significant difference between the 1-2 clusters' means ($p=0.438$). 'Porose areas height/width ratio' variable did not present a statistically significant difference between the 2 and the rest of the clusters' means ($p=0.888$, 0.929 and 0.939 relatively to the clusters 1, 3 and 4 means). 'Spiracle Oval area height/width ratio' variable did not

present a statistically significant difference between the 1-3, 1-4 and 3-4 clusters' means, (p=0.359, 0.836, and 0.067 respectively).

Therefore, the variables that significantly distinguish more clusters were the 'Scutum length/width ratio' and the 'Spiracular angle' variables; and the ones that significantly distinguish fewer clusters were the 'Basis capituli length/width ratio' and the 'Porose areas height/width ratio' variables. The 'Basis capituli length/width ratio' was although useful for the formation of the cluster 2, based on the means obtained before.

- ***Qualitative variables clusters characterization***

To classify the formed females' qualitative variables clusters, from the 137 studied specimens of this gender, a cross-tabulation statistics was performed. The qualitative variables clusters characterization by percentage it is describe for each cluster in the pg. 128, Appendix III, Section I.

In order to classify the statistical significance of this results, the association measures Cramer's V and Chi-square test results were analysed.

The 'scutum posterior margin shape' variable present the results V =0.434, and result in $\chi^2(1)=51.695$ with a p=0.000 (which was the only qualitative variable in chi-square test conditions to be correctly interpreted – less than 20% of cells have expected count less than 5, and the minimum expected count is 1). This means that there is a strong relationship between the variables, or in other words, there is a statistically significant large effect of the variable on the qualitative clusters formation.

The 'Second palp shape' variable result in V=0.482. This means that the variable has a strong relationship with the qualitative group's variable, that is, the 'second palp shape' variable had a large effect on the qualitative clusters formation. The 'scutum punctation distribution' variable result in V =0.199, which tell us that this variable has a low effect on the clusters formation. The 'scutum punctation size' variable result in V=0.689, which means that the variable have a very strong relationship with the clusters formation, and being the highest V-value obtained, it is the main variable for the 'qualitative variables groups' formation. The 'cervical fields shape' variable obtained the results V =0.323, which means that the relationship between variables is strong, that is, have a medium effect on the qualitative clusters formation. The 'setiferous punctations size' variable

result in V =0.382, which means that the relationship between variables is strong, and so have a high effect on the qualitative clusters formation. For last, the 'cervical grooves definition' variable obtain the values V =0.138, which means that the variables have a weak relationship, which is translated in a low effect of this variable in qualitative clusters formation.

Therefore, we obtain that the 'scutum punctation size' variable is the main base for the groups formation, the 'second palp shape' variable has the second strongest effect on the cluster formation, followed by the 'scutum posterior margin shape' variable, the 'setiferous punctations size' variable have some effect on the groups formation, and the 'cervical fields shape' variable have a lower effect when compared to the others but with a strong effect on the 'qualitative variable groups' formation.

These variables were all weak on the global separation of the groups, less the 'scutum punctation size' variable that separate clusters with different proportions of the qualitative variables.

- **Correspondence Analysis**

The CA is a multivariate statistical technique which looks into the association of two or more categorical variables and displays them jointly on a bivariate graph. It allows data reduction and graphical representation of dissimilarities on categorical variables.

This statistic technique were then applied to qualitative variables and to quantitative variables clusters formed before, so that we might draw conclusions regarding associations among the clusters based on the two different variables. As the inertia value obtained for a first analysis was I=0.065 (total above 0.20 is expected for adequate representations) and the chi-square result is not statistically significant ($\chi^2(1)=8.951$, p=0.442), the analysis of this output (Fig.24) is not regarded as acceptable, showing a weak correlation between variables. Then, we proceed by deduct the more outlier cluster, and as can be seen in the Fig.24, it is the qualitative variables cluster 2. The obtained final result is in Fig.25.

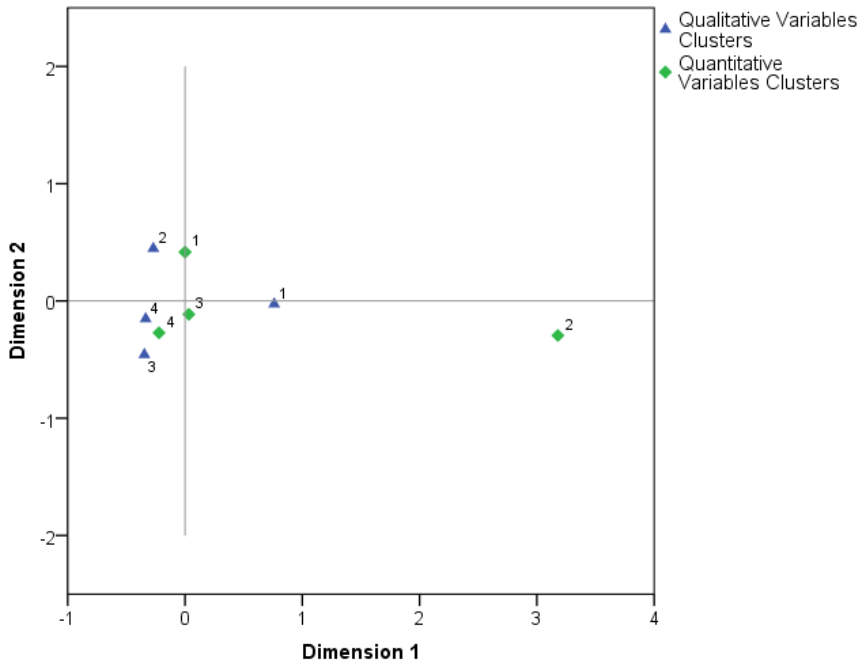

Fig.24 – Bivariate graph obtained from correspondence analysis of the qualitative variables with the quantitative variables of females formed clusters. It can be observed that the cluster 2 of the "Qualitative variables clusters" did not seem to have any association with the other clusters. In other hand, both 4 and both 3 clusters have some association, as is the 2 and 1 clusters from the qualitative and quantitative variables clusters categories, respectively. But as the inertia and the chi-square results (I=0.065, p=0.442) showed that this correlation is not statistically significant and is weak, this associations referred before have to be revaluated.

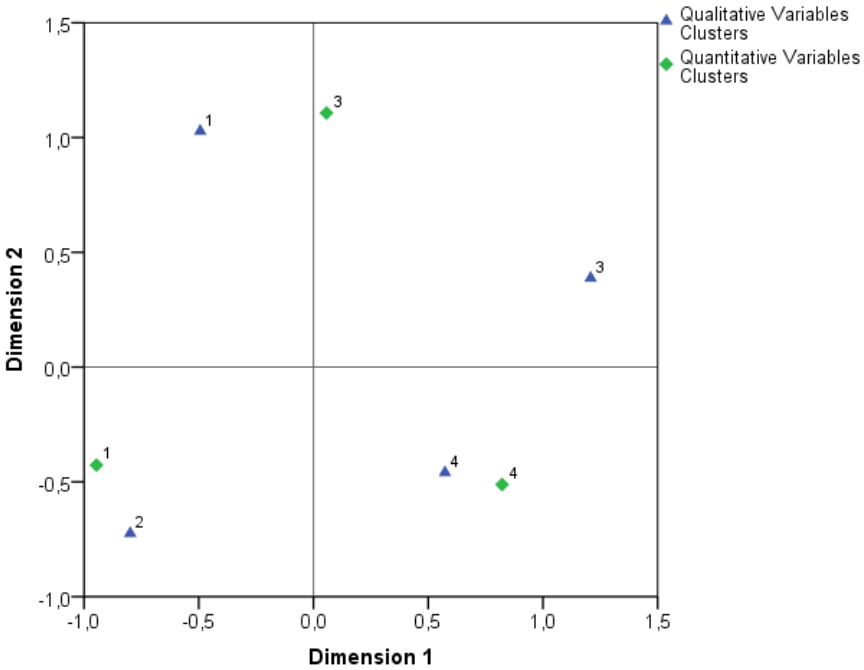

Fig.25 – Bivariate graph obtained from Correspondence Analysis displaying the qualitative variables and the quantitative variables of females clusters, excluding the cluster 2 of the last category, and how they relate to each other on two dimensions. Inertia value obtained in this analysis, I=1.070, is regarded as an acceptable strong correlation one. The new dimensions allow us to evidence the associations between clusters 1-3 (correspondent to qualitative and quantitative typologies, respectively); 2-3 (qualitative and quantitative typologies, respectively); 4-4 (qualitative and quantitative typologies); and the cluster 3 of the qualitative typology have association with 3 and the 4 clusters, both of the quantitative typology.

The new analysis showed an inertia value of I=1.070, which means that is a strong and acceptable correlation between the categorical variables in analysis. As one pair of categories is such more similar the more closely are their projections in the graphic representation of CA, the interpretation of the Fig.25 biplot shows the following associations: between cluster 1 (of the qualitative typology) and cluster 3 (quantitative typology); among cluster 2 (qualitative typology) and cluster 1 (quantitative typology); between 4 cluster (typology of qualitative) and cluster 4 (type of quantitative); and the third cluster (of the qualitative typology) associates itself to both the cluster 3 and cluster 4 (of the quantitative typology).

It can be said that the cluster 1 of the quantitative variables and the cluster 2 or the qualitative variables share the following common characteristics: high spiracular angles (more linear spiracles), higher palps with square and long in width shapes, smooth and slightly sinuous posterior margin of the scutums, small punctation size, and a small basis capituli. So, probably these features are usable to describe one cluster of elements with more common morphological features.

The cluster 1 of the qualitative variables and cluster 3 of the quantitative variables present elements with low spiracle angles (which it translates in more globular spiracles, with an up pointed tail), short palps with square and long in with shapes, basis capituli with an intermediate size, small and medium (or just small) punctations, smooth, slightly sinuous and sinuous posterior margins of the scutums.

The associate clusters 4 of both typologies are characterized by specimens with the bigger basis capituli, high and long in width palps, with large spiracle angle (more linear spiracles), small punctations, and slightly sinuous and sinuous posterior margins of the scutum.

- ***Taxonomic Groups Correspondence Analysis***

Next, both qualitative and quantitative variables clusters categories were compared by CA with the taxonomy groups obtain from the traditional morphological evaluation of the female specimens. The bivariate final graphs obtained (Fig.26, Fig.27 and Fig. 28) are shown next.

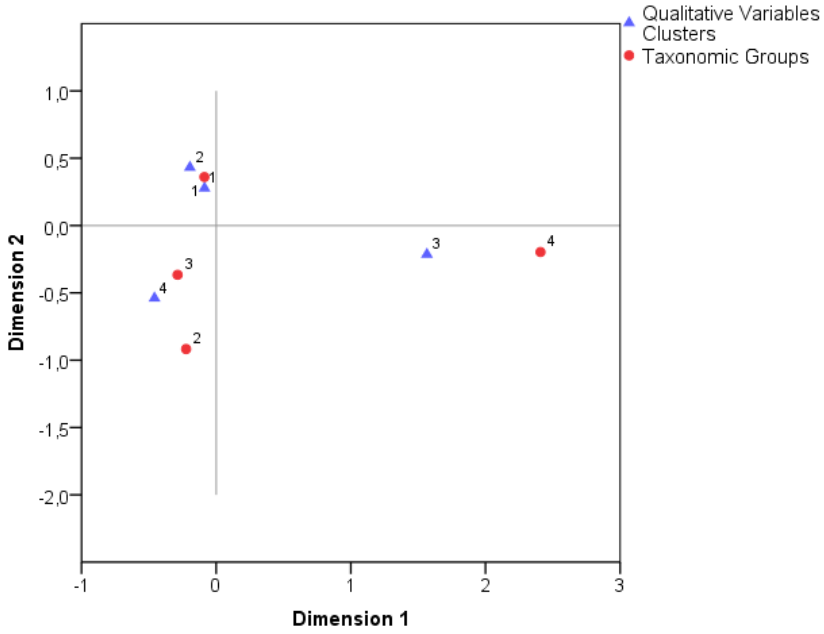

Fig.26 – Bivariate graph obtained from Correspondence Analysis displaying the females qualitative variables and their taxonomic groups, and how they relate to each other on two dimensions. In this analysis, I=0.206 and p=0.001, which is regarded as an acceptable significant but weak correlation. The new dimensions allow us to evidence the associations between clusters 3-4 (correspondent to qualitative and taxonomic typologies, respectively); 2-1 (correspondent to qualitative and taxonomic typologies, respectively); 1-1 (correspondent to qualitative and taxonomic typologies); 4-3 (correspondent to qualitative and taxonomic typologies, respectively); and also 4-2 (correspondent to qualitative and taxonomic typologies, respectively), being this last one association weaker than the of the 4-3 (correspondent to qualitative and taxonomic typologies, respectively).

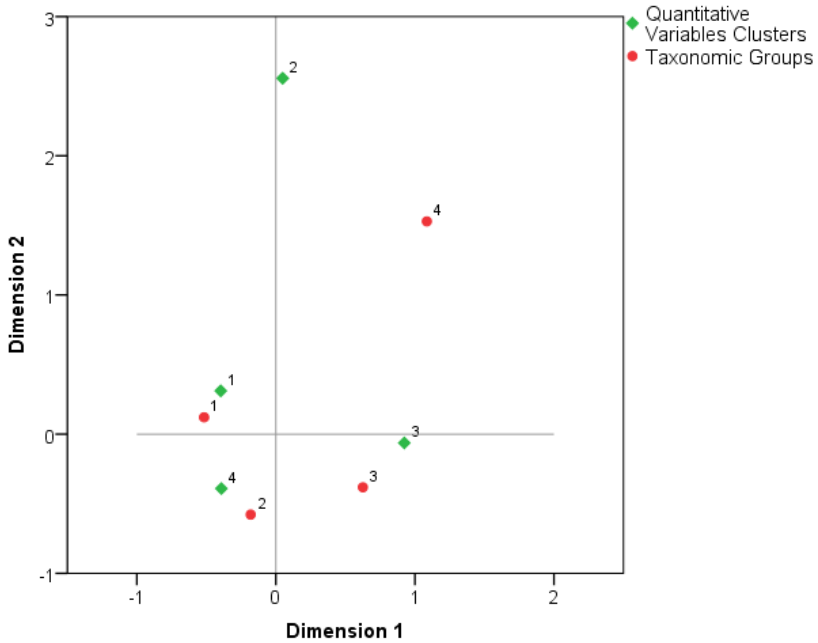

Fig.27 – Bivariate graph obtained from Correspondence Analysis displaying the females quantitative variables and their taxonomic groups, and how they relate to each other on two dimensions. In this analysis, I=0.181 and p=0.003, which is not regarded as an acceptable significant correlation. Even so, the new dimensions allow us to evidence the association between clusters 2-4 (correspondent to quantitative and taxonomic typologies, respectively).

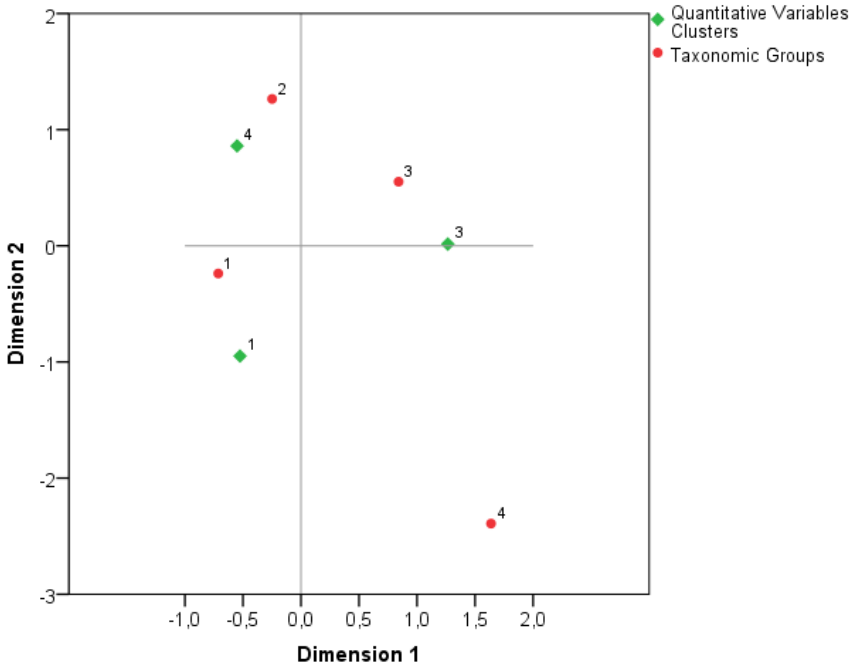

Fig.28 – Bivariate graph obtained from Correspondence Analysis displaying the females quantitative variables and their taxonomic groups, and how they relate to each other on two dimensions. In this analysis, I=1.255, which is regarded as an acceptable significant correlation. The new dimensions allow us to evidence the association between clusters 4-2 (correspondent to quantitative and taxonomic typologies, respectively), 1-1, and 3-3(correspondent to quantitative and taxonomic typologies).

In the CA between the qualitative variable clusters and the taxonomic groups (see Fig.26), I=0.206 and p=0.001, which is regarded as an acceptable and statistically significant but weak correlation. The new dimensions allow us to evidence the associations between clusters 2-1 (correspondent to qualitative and taxonomic typologies, respectively); 1-1 (correspondent to qualitative and taxonomic typologies); 4-3 (correspondent to qualitative and taxonomic typologies, respectively); and also 4-2 (correspondent to qualitative and taxonomic typologies, respectively). The 3-4 cluster association (correspondent to qualitative and taxonomic typologies, respectively) is the weakest of the weaker associations, so it result will be not significant. The 4-3 (correspondent to qualitative and taxonomic typologies, respectively) association is stronger than the 4-2 (correspondent to qualitative and taxonomic typologies, respectively) cluster association, regarding the distances between the groups presented on the biplot.

This tell us that the taxonomic group 1, equivalent to *R. sanguineus* specimens, have the characteristics in common with qualitative clusters 1 and 2, this is, square and long in width palps, small and medium punctations, and all the posterior margin shapes described in this study. This results are not very conclusive, and with weak association, but can be said that within this group are still present much features variations.

The same happens with the taxonomic groups 2 and 3, that were not been differentiated, but were characterized has having elements with long in with palps, small punctations, slightly sinuous and sinuous margins. This result said that the intermediate cluster formed and the *R. turanicus* cluster has more in common than with the *R. sanguineus* cluster. But complicating the matter further, the taxonomic clusters 1, 2 and 3 (*R. sanguineus, R. turanicus* and intermediate) have more in common than the cluster 4 (*R. pusillus*), which is a correct correlation result.

In the CA between the quantitative variable clusters and the taxonomic groups (see Fig.27), was obtain that I=0.181 and p=0.003, which is not regarded as an acceptable significant correlation. As done before, the cluster 2 of the quantitative variables are given as missing to be possible to procedure with the analysis. Even so, the new dimensions allow us to evidence the association between clusters 2-4 (correspondent to quantitative and taxonomic typologies, respectively). In this new analysis (see Fig.28), I=1.255, which is regarded as an acceptable significant correlation. The new dimensions allow us to evidence the association between clusters 4-2 (correspondent to quantitative and taxonomic typologies, respectively), 1-1, and 3-3 (correspondent to quantitative and taxonomic typologies).

This means that the intermediate cluster 2 (taxonomic typology) have in common with cluster 4 (quantitative variables) the characteristics: big basis capituli, high palps and big scutums. The *R. turanicus* cluster and the cluster 3 present elements with shorter palps, low spiracle angles and low ratio of widths measured in the same feature, what means that they have small and globular spiracles, with probably small and more linear tails. They are differentiated from the cluster 1 (*R. sanguineus*) because, using its association with cluster 1 of the quantitative variables, just have a conclusive result relatively to be characterize by a small basis capituli. Again, it can be concluded that the *R. sanguineus* group can be well defined because present to much intra-specific variation, and the solution is to create more groups of variability to accommodate it. The cluster *R. turanicus* characteristics are in accordance with the describe characteristics for this species.

The exclusion of the cluster 4 (taxonomic typology), referent to *R. pusillus*, of the association distance help us to conclude that this results are statistically right and are significant.

4.2.2. Males Morphological Features Analysis

Of 289 males analysed, 58 (20.1%) of them were collected in Óbidos, 200 (69.2%) in Santarém, and 31 (10.7%) in Caldas da Rainha districts. As already referred before, in this study, we are not going to take into account the proportions of the different species in different districts due to the low number of samples in two of them, being this description just an apart observation.

- **Traditional taxonomic analysis**

Traditional taxonomic analysis was performed, and four clusters were formed: (1) *R. sanguineus* cluster, (2) Intermediary cluster, (3) *R. turanicus* cluster, (4) *R. pusillus* cluster. The intermediary cluster is characterized by specimens which has some morphological features that resembles *R. sanguineus* and others that resembles *R. turanicus* species. *R. pusillus* specimens were included in the analysis as an outlier group for comparison purposes.

Of the 289 males specimens in the study, 221 (76.5%) were identified as *R. sanguineus*, 38 (13.1%) were identified as intermediate, 25 (8.7%) were identified as *R. turanicus*, and 5 (1.7%) were identified as *R. pusillus*. These results are shown in Fig.29.

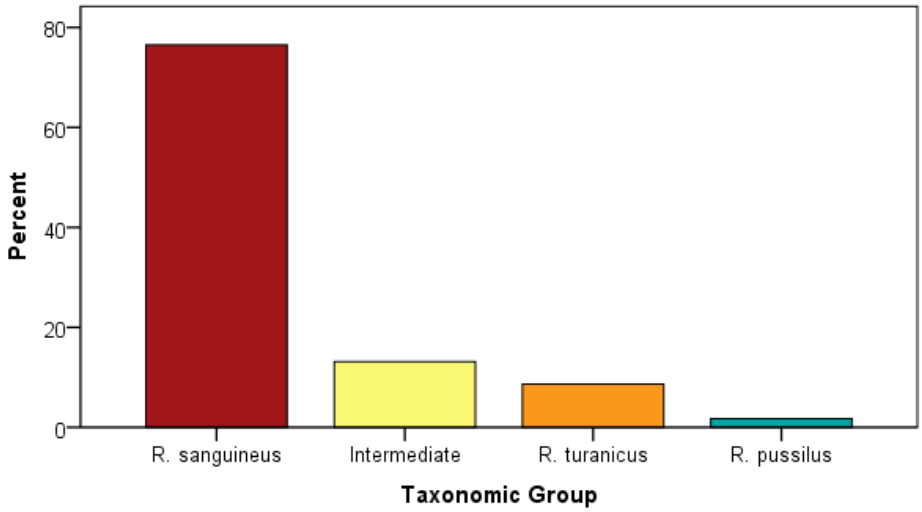

Fig.29 – Graphic representation of the specimens' percentage per taxonomic group formed. Of the 289 males specimens in the study, 221 (76.5%) were identified as *R. sanguineus*, 38 (13.1%) were identified as intermediate, 25 (8.7%) were identified as *R. turanicus*, and 5 (1.7%) were identified as *R. pusillus.*

▪ Hierarchical Cluster Analysis

Then, Hierarchical Cluster Analysis on SPSS software was performed in separate for quantitative variables and for qualitative analysis. As in a first analysis we obtain a cluster with just one element, that specimen (ID=S1236, ZC/IICT) were taken out for the purpose of not disturb the results, and will be latter analysed. The obtained dendrograms are in Fig.30 and Fig.31.

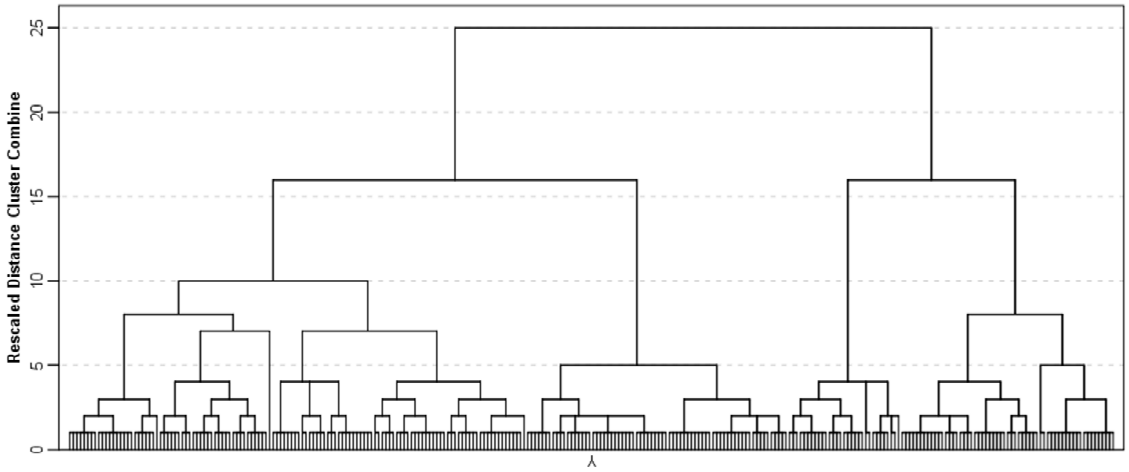

Fig.30 – Hierarchical Cluster Analysis dendrogram obtained with males' quantitative variables data. The higher distance between fusion coeficients were obtained in the rescaled distance value 15 (forming 4 clusters).

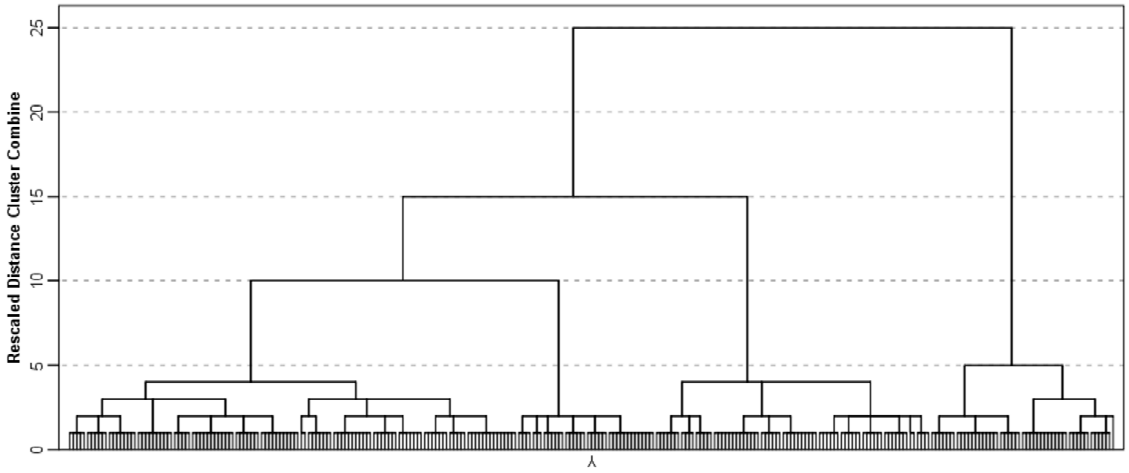

Fig.31 – Hierarchical Cluster Analysis dendrogram obtained with males' qualitative variables data. The higher distance between fusion coeficients were obtained in the rescaled distance value 7 (forming 4 clusters).

Based on the fusion coefficients distances obtained during the cluster analysis (Appendix II, pg. 127, Table I.1), we chosen four clusters to be formed in both analysis for quantitative and qualitative variables. This choice is in accordance with the data observed in the dendrograms of Fig.30 and Fig.31.

Thereafter, and with a cluster perfil characterization purpose, ANOVA statistical model and cross-tabulation statistics was performed for quantitative and qualitative variables clusters, respectively.

- ***Quantitative Variables Clusters Analysis***

To classify the formed males' quantitative variables clusters characteristics, a one-way ANOVA statistical analysis was performed. The table referent to the descriptive measures of the variables within the clusters is Table.9.

The mean was the statistical descriptive measure used to describe the clusters formed by the males' quantitative variables. The Fig.32 and Fig.33 are graphically represented the means of the quantitative variables within the 4 clusters formed. Again, as in the females' case, the 'Spiracular angle' quantitative variable showed the highest unit values (measured in angle degrees), and consequently highest standard deviation between clusters in comparison to the others quantitative variables included on the study. For more detailed data, see Fig.32 and Table.9.

Table.9 – Males descriptive statistics of quantitative variables within the clusters formed by hierarchical cluster analysis. All measures were taken in millimeters, less the angle, taken in angle degrees. The specimen ID=CZ S1236 (IICT) is not included. N- number of elements within the clusters, Std. Deviation - standard deviation.

Morphological feature	Cluster	Descriptives Measures				
		N	Mean	Std. Deviation	Minimum	Maximum
Ventral-Measured Palp height	1	59	0,366	0,024	0,315	0,433
	2	126	0,369	0,046	0,245	0,515
	3	31	0,342	0,036	0,242	0,408
	4	72	0,373	0,032	0,302	0,463
	Total	288	0,367	0,039	0,242	0,515
Spiracular angle	1	59	142,276	13,201	111,693	169,521
	2	126	144,657	14,082	99,705	178,456
	3	31	141,364	14,691	120,511	177,198
	4	72	123,299	14,066	77,345	151,068
	Total	288	138,475	16,478	77,345	178,456
Conscutum lenght/width ratio	1	59	1,837	0,077	1,680	1,998
	2	126	1,684	0,089	1,390	1,920
	3	31	1,731	0,083	1,614	1,891
	4	72	1,726	0,076	1,549	1,893
	Total	288	1,731	0,100	1,390	1,998
Basis Capituli lenght/width ratio	1	59	0,869	0,053	0,727	0,960
	2	126	0,826	0,062	0,649	0,976
	3	31	0,871	0,068	0,673	0,964
	4	72	0,888	0,044	0,788	0,992
	Total	288	0,855	0,062	0,649	0,992
Spiracle Oval Area lenght/width ratio	1	59	2,505	0,414	1,708	4,440
	2	126	2,381	0,263	1,847	3,335
	3	31	2,105	0,243	1,601	2,985
	4	72	2,468	0,202	1,882	2,967
	Total	288	2,398	0,307	1,601	4,440
Spiracular area third-widths ratio	1	59	1,557	0,288	0,543	2,121
	2	126	1,805	0,252	1,264	2,394
	3	31	1,415	0,188	0,976	1,726
	4	72	1,616	0,212	1,159	2,160
	Total	288	1,665	0,278	0,543	2,394
Adanal Plates height/width ratio	1	59	2,269	0,266	1,650	2,953
	2	126	2,219	0,310	0,371	3,237
	3	31	2,223	0,238	1,824	2,746
	4	72	2,204	0,170	1,775	2,531
	Total	288	2,226	0,264	0,371	3,237
Spiracular tail final width/adjacent festoon width ratio	1	59	0,264	0,073	0,124	0,436
	2	126	0,252	0,078	0,106	0,489
	3	31	0,504	0,145	0,236	0,837
	4	72	0,230	0,069	0,125	0,419
	Total	288	0,276	0,116	0,106	0,837
After posteromedian grooves measured width/conscutum width ratio	1	59	0,821	0,050	0,695	0,921
	2	126	0,907	0,064	0,695	1,031
	3	31	0,862	0,055	0,756	0,978
	4	72	0,932	0,050	0,800	1,024
	Total	288	0,891	0,070	0,695	1,031

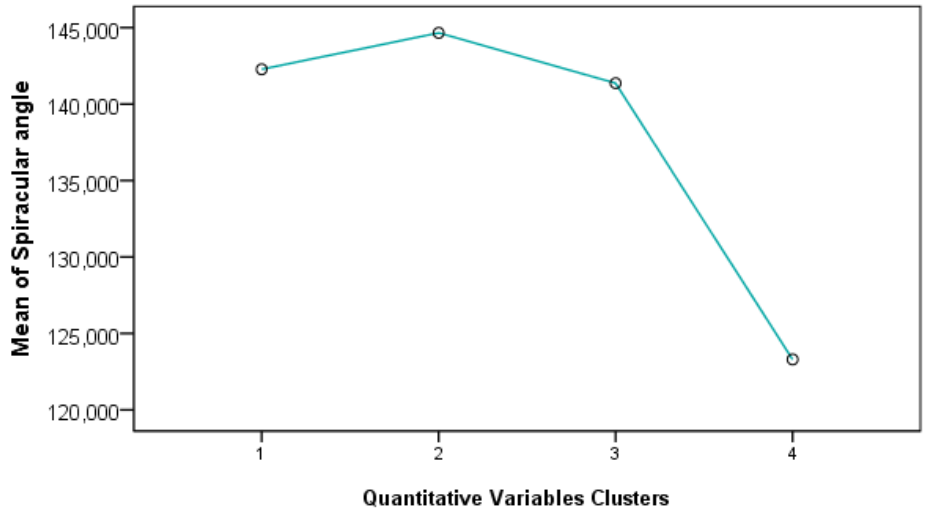

Fig.32 – "Spiracular angle" quantitative variable males' clusters means. This variable present the highest mean values (unit degree) in comparison to the other quantitative variables. The only cluster to have a clearly different mean, in comparison for the others, is the cluster 4. The clusters 1 and 3 are hardly differentiated by this descriptive measure. Clusters means: Cluster 1 – μ=142.276, Cluster 2 – μ=144.657, Cluster 3 – μ=141.364, and Cluster 4 – μ=123.299.

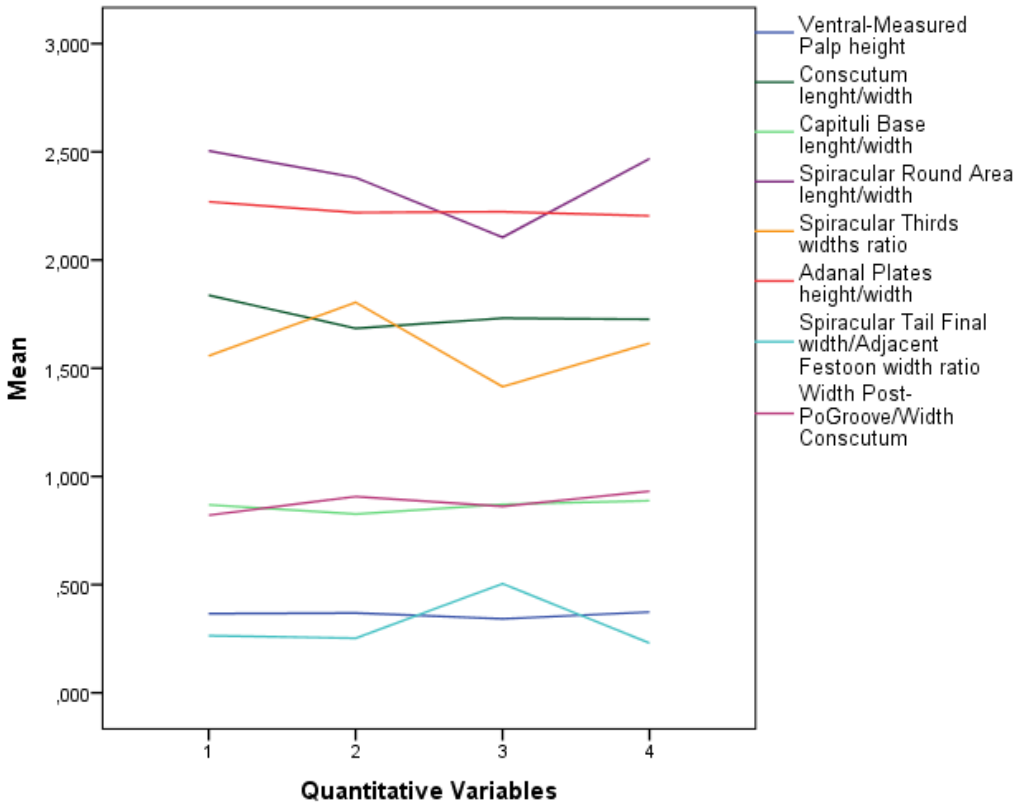

Fig.33 – Clusters means obtained based on all males' quantitative variables less the "Spiracular angle" quantitative variable. The 'Width post-pogroove/width conscutum', the 'Spiracular area thirds widths ratio', and the 'Spiracle Oval area length/width' variables are the ones where the means clearly defines the different clusters, presenting the highest standard deviations by a descending order. The follow variables did not differentiated the means two clusters: 'Ventral-measured palp height' of the 1-2 clusters, 'Conscutum length/width ratio' of the 3-4 clusters, 'Basis Capituli length/width ratio' of the 1-3 clusters, 'Adanal Plates height/width ratio' of the 2-3 clusters. . The follow variables did not differentiated the means three clusters: 'Spiracular angle' of the 1-2-3 clusters, and 'Spiracular tail final width/adjacent festoon width ratio' of the 1-2-4 clusters.

By the evaluation of Table.9, Fig.32 and Fig.33, in contribution descending order to the differentiation between clusters, we have:

- The ‘After posteromedian grooves measured width/conscutum width ratio’, the ‘Spiracular area thirds-widths ratio’, and the ‘Spiracle Oval area length/width ratio’ variables that are the ones where the means clearly defines the different clusters;
- The ‘Ventral-measured palp height’, the ‘Conscutum length/width ratio’, the ‘Basis Capituli length/width’, the ‘Spiracular angle’, and the ‘Adanal Plates height/width ratio’ variables did not differentiated the means of 1-2, 3-4, 1-3, 1-3 and 2-3 clusters, respectively;
- The ‘Spiracular tail final width/adjacent festoon width ratio’ variable did not differentiated the means of 1-2-4 clusters, being this the variable that have the lowest contribute to the clusters differentiation by its means.

Using these results, can be said that:

- The cluster 1 has ticks with conscutums larges in length, but thinner in widths; with big spiracles with medium angles, and final widths of the tail smaller than the adjacent festoon width; the biggest adanal plates present in the study population, and medium palps height;
- The cluster 2 has elements characterized by short conscutums in length but large in width; large spiracles with a big angle, final widths of the spiracular tail smaller than the adjacent festoon width, medium palps highs, small basis capituli, and medium adanal plates;
- The cluster 3 has specimens with average size conscutum, short palps, medium basis capituli and adanal plates, and small spiracles with medium angles and with large final width of the tail (as large as the adjacent festoon);
- The cluster 4 has elements with a very broad but not too long conscutum, high palps, big capituli basis, small adanal plates, spiracular small angles, with final widths of the tail smaller than the adjacent festoon width, and not very larges.

In order to classify the statistical significance of the results, we proceeded with an ANOVA statistical analysis. The follow values were obtained:

The ‘Adanal Plates height/width ratio’ variable result in F=0.712 and p=0.545, this is, the variable did not statistical significantly differentiated the between cluster’ means; the ‘Spiracular tail final width/adjacent festoon width ratio’ variable result in F=85.760 and p=0.000, the ‘After posteromedian grooves measured width/conscutum width ratio’ variable result in F=47.262 and p=0.000, the ‘Conscutum length/width ratio’ variable result in F=46.118 and p=0.000, the ‘Spiracular angle’ variable result in F=34.454 and p=0.000, the ‘Spiracular area thirds-widths ratio’ variable result in F=29.296 and p=0.000, the ‘Basis Capituli length/width ratio’ variable result in F=20.689 and p=0.000, the ‘Spiracle Oval area length/width ratio’ variable result in F=15.142 and p=0.000, the ‘Ventral-measured palp height’ variable result in F= 5.298 and p=0.001. Therefore, all this variables statistical significantly differentiated the cluster’ means, that is, all of them contribute significantly for the clusters formation. Based on the F-value, the variables are in descending order of significance for the cluster formation, this is, the ‘Spiracular tail final width/adjacent festoon width ratio’ variable was the one that gave the biggest contribute to the cluster 3 differentiation (the only one that this variable differentiated), and the ‘Ventral-measured palp height’ variable was the one that less significantly contributed for the clusters formation.

Relatively to the multiple comparison Tukey HSD test (post hoc test), which is a single-step multiple comparison procedure normally used in conjunction with ANOVA statistical tool to find means that are significantly different from each other, statistically significant p-values were observed. For note, and has the ‘Adanal Plates height/width ratio’ variable did not result in the exclusion of the H_0 hypothesis (p>0.050), it cannot be evaluated by this test and is not statistically significant for the differentiation of the clusters’ means.

The ‘Conscutum length/width ratio’ variable did not present a statistically significant difference between 3-4 cluster’ means (p=0.993). The ‘Spiracular area thirds-widths ratio’ variable did not present a statistically significant difference between 1-4 cluster’ means (p=0.530). The ‘Spiracle Oval area length/width ratio’ variable did not present a statistically significant difference between 1-4 and 2-4 clusters’ means (p=0.888 and 0.169 respectively). The ‘Ventral-measured palp height’ variable did not present a statistically significant difference between 1-2, 1-4, and 2-4 clusters’ means (p=0.973, 0.714, and 0.856 respectively). The ‘Spiracular angle’ variable did not present a statistically significant difference between 1-2, 1-3, and 2-3 clusters’ means (p=0.702,

0.991, and 0.643 respectively). The 'Basis Capituli length/width ratio' variable did not present a statistically significant difference between 1-3, 1-4, and 3-4 clusters' means (p=0.998, 0.231, and 0.516 respectively). The 'Spiracular tail final width/adjacent festoon width ratio' variable did not present a statistically significant difference between 1-2, 1-4, and 2-4 clusters' means (p=0.815, 0.103, and 0.285 respectively).

The 'After posteromedian grooves measured width/conscutum width ratio' variable differentiate with significance all the clusters' means (all $p<0.05$), giving the highest contribute to the clusters formation.

- ***Qualitative Variables Clusters Analysis***

To classify the formed males' qualitative variables clusters, from the 288 studied specimens of this gender, a cross-tabulation statistics was performed. The males' qualitative variables clusters characterization by percentage it is described for each cluster in Appendix III, pg.128, Section II.

Following, association measure Cramer's V and Chi-square test results (only displayed when conditions to be interpreted are full field) were obtained for all variables relatively to the qualitative variables groups:

- The 'Cervical fields depression' variable result in V=0.908 and p=0.000 ($\chi^2(1)=237.424$), which tell us that this variable has a statistically significant very strong relationship and being the highest V-value obtained, it is the main variable for the 'qualitative variables groups' formation.
- The 'Parma presence' variable result in V=0.890 and p=0.000 ($\chi^2(1)=227.955$), which tell us that this variable has a statistical significant super large effect on the qualitative clusters formation, but with a low effect then the 'Cervical fields depression' variable.
- The 'Adanal plates posterior margin' variable result in V=0.696 and p=0.000 ($\chi^2(1)=139.530$), and the 'Cervical fields shape' variable result in V=0.573 and p=0.000 ($\chi^2(1)=284.100$), which are statistically significant results and very large effects on the qualitative clusters formation (written by descending order).
- The 'Cervical grooves definition' variable result in V=0.425 and p=0.000 ($\chi^2(1)=52.072$), and the 'Adanal plates Ending' variable result in V=0.419 and p=0.000

($\chi^2(1)=50.668$), which are a moderate statistical significant effects on the qualitative clusters formation.

- The 'Posteromedian grooves shape' variable result in V=0.145 and p=0.034 ($\chi^2(1)=18.104$), which tell us that this variable has a low statistical significant effect on the qualitative clusters formation.
- The 'Lateral grooves beginning' variable result in V=0.156 and p=0.072 ($\chi^2(1)=7.001$), and the 'Paramedian grooves deepness' variable result in V=0.152 and p=0.083 ($\chi^2(1)=6.670$), which are low and not statistical significant effects on the qualitative clusters formation (by descending order).
- The 'Posteromedian grooves deepness' variable result in V=0.098 and p=0.431 ($\chi^2(1)=2.756$), and the 'Posteromedian grooves length' variable result in V=0.068 and p=0.724 ($\chi^2(1)=1.321$), which are little and not statistical significant effects on the qualitative clusters formation.
- The next variables did not had a significant effect on the clusters qualitative formation: the 'Adanal plates total shape' variable result in V=0.511, which has a moderate effect on the clusters formation; the 'Second palp shape' variable result in V=0.180, the 'Lateral grooves texture' variable result in V=0.134, the 'Conscutum punctation size' variable result in V=0.127, the 'Cervical fields setiferous punctations' variable result in V=0.124, the 'Conscutum punctation distribution' variable result in V=0.119, and the 'Lateral grooves festoons ending' variable result in V=0.105, which tell us that this variables have a low effect on the qualitative clusters formation (variables are written in descending order of effect).

▪ Correspondence Analysis

The CA was then applied to qualitative variables and to quantitative variables clusters formed before, so that we might draw conclusions regarding associations among the males' clusters based on the two different variables.

As the inertia value obtained for the first analysis was I=0.244, with p=0.000 as chi-square result, it is regarded as a significant strong correlation between the both variables (Fig.34).

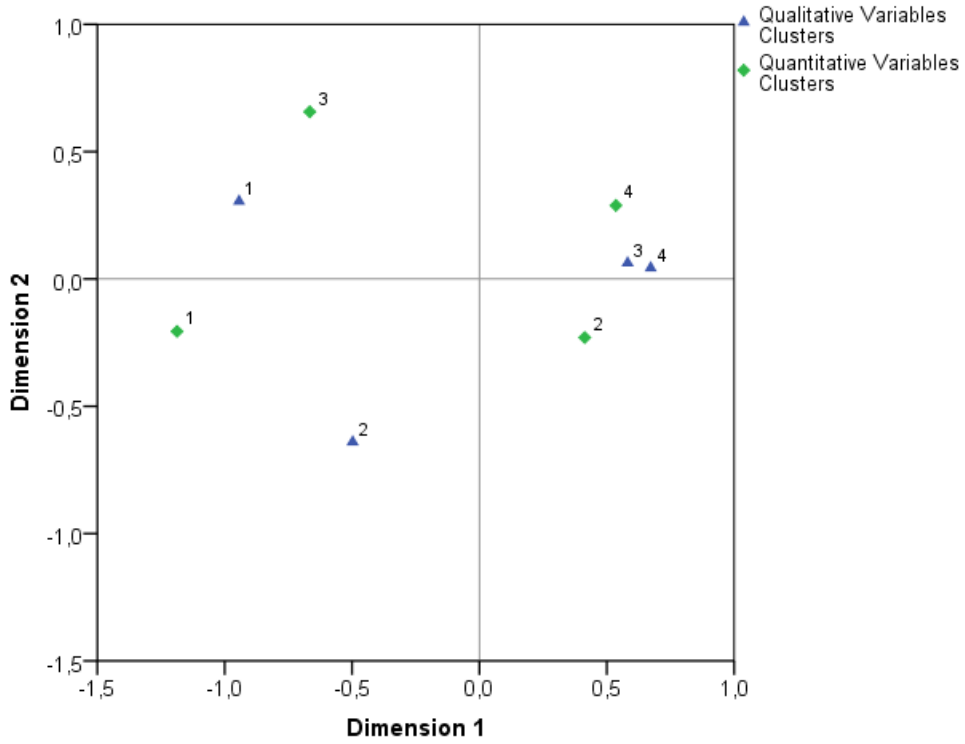

Fig.34 – Bivariate graph obtained from correspondence analysis of the males' qualitative variables with the quantitative variables formed clusters. As I=0.244 and p=0.000 were obtained as result of this correlation analysis, the clusters associations obtained from it are considerate significant and strong ones. Clusters association: 3B-4G, 4B-4G, 3B-2G, 4B-2G, 1B-3G, 1B-1G, 2B-1G (in descending order of association). It is to note that cluster 3 and 4, originated from the same variables analysis, were not supposed to have a close association, so this clusters are not well characterized by this variables. B-blue marker, G-green marker.

The interpretation of the Fig.34 biplot shows the following associations between clusters: 3-4 (of the qualitative and quantitative typology, respectively), 4-4 (of the qualitative and quantitative typology), 3-2 (of the qualitative and quantitative typology, respectively), 4-2 (of the qualitative and quantitative typology, respectively), 1-3 (of the qualitative and quantitative typology, respectively), 1-1 (of the qualitative and quantitative typology, respectively), and 2-1 (of the qualitative and quantitative typology, respectively).

Moreover, it is possible to conclude that the clusters 1 blue (qualitative variables) and 3 green (quantitative variables) have both animals with apparent cervical fields depression, absent parma, square and round adanal plates posterior margin, average size conscutum, short palps, and small spiracles with medium angles and with large final width of the tail (as large as the adjacent festoon); and the clusters 1 green (quantitative variables) and 2 blue (qualitative variables) present animals with absent cervical depression, more square than round adanal plates posterior margin, absent and small cervical fields shape, conscutums larges in length but thinner in widths, the biggest adanal plates, big spiracles with final widths of the tail smaller than the adjacent festoon width.

As the other 4 clusters were too associated to be differentiated, especially those of the qualitative variables analysis, clusters 3 and 4, which are too similar to be correctly characterized. However, these results are consistent with reported fact that if more clusters are formed, it will be possible to achieve more conclusive results regarding the variety showed.

- ***Taxonomic Groups Correspondence Analysis***

Following, both qualitative and quantitative variables clusters categories were compared by correspondence analysis with the taxonomy groups obtain from the traditional morphological evaluation of the female specimens. The bivariate graphs obtained are shown next in Fig.35 and Fig.36 (pg.79).

In the CA between the qualitative variable clusters and the taxonomic groups (Fig.35, pg.79), with the results I=0.075 and chi-square p=0.010, is regarded as a very weak but statistically significant analysis of clusters association. The new dimensions allow us to evidence the associations between the clusters: 3-1, 1-4, 1-3, and 2-2 (in all the cases, clusters are written in the order qualitative and quantitative typology). Clearly, the 4 cluster of the taxonomic groups are isolated from the others in the fourth quadrant, being that indicative of good associations between clusters, because this cluster is correspondent to the control group *R. pusillus* (morphologically different from *R. sanguineus*), as already mentioned.

This means that the qualitative variables clusters 4 and 3 are associated with *R. sanguineus* taxonomic cluster, which is in concordance with the results obtain from CA of the both variables typologies, evidencing the big intra-variability present in *R. sanguineus* specimens. The qualitative variables cluster 1, for is hand, is identical to *R. turanicus* cluster, being characterized for not having parma, having apparent cervical fields depression and square and round adanal plates. And for last, the qualitative variables cluster 2 have more in common with the intermediate taxonomic cluster, being interesting to note that this group are significantly different from the others, demonstrating a good start to unravel the *R. sanguineus* group, being characterized by elements which have absent cervical depressions, more square than round posterior margins of the adanal plates, and absent or small cervical fields shape.

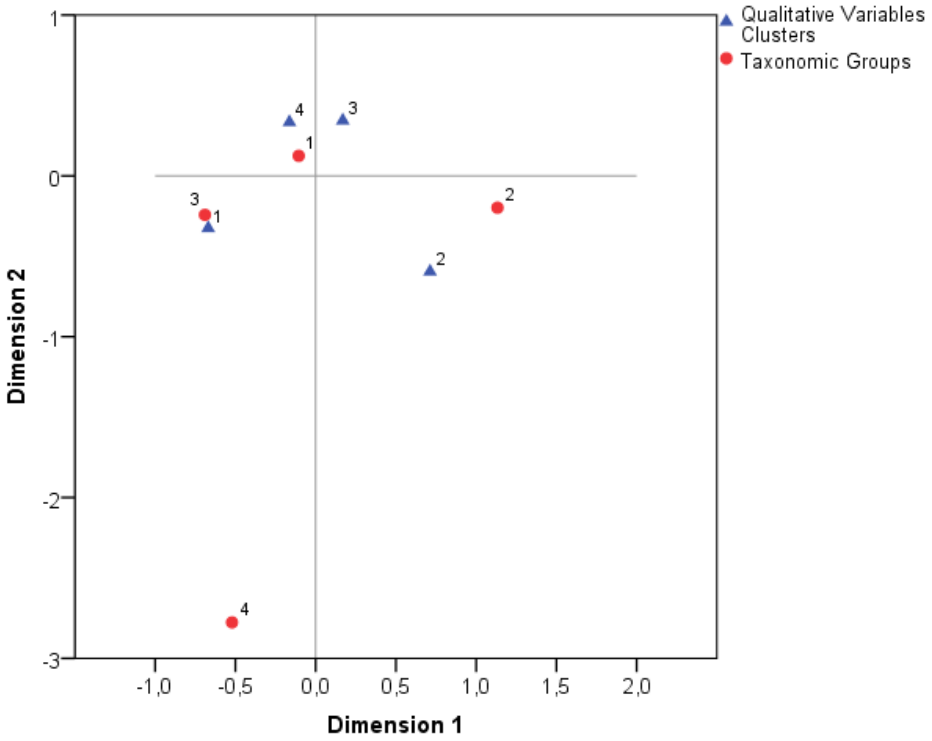

Fig.35 – Bivariate graph obtained from Correspondence Analysis displaying the males' qualitative variables and the taxonomic groups, and how they relate to each other on two dimensions. As I=0.075 and p=0.015 were obtained as result of this correlation analysis, this clusters associations are significant but weak. Clusters association: 3R-1B, 1R-4B, 1R-3B, 2R-2B (in descending order of association). The 4R cluster is clearly isolated. R-red marker, B-blue marker.

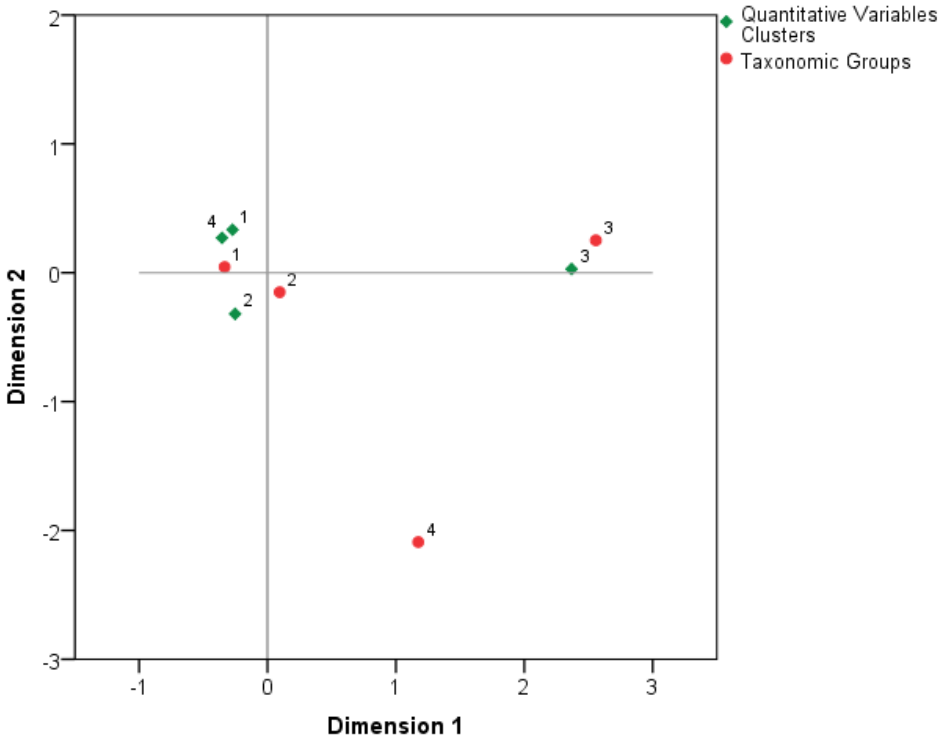

Fig.36 – Bivariate graph obtained from Correspondence Analysis displaying the males' quantitative variables and the taxonomic groups, and how they relate to each other on two dimensions. As I=0.467 and p=0.000 were obtained as result of this correlation analysis, being this clusters associations considerate significant and strong related. Clusters association: 1R-4G 1R-1G 1R-2G 2R-1G 3R-3G (in descending order of association). The 4R cluster is clearly isolated. R-red marker, G-green marker.

Relatively to the quantitative variable clusters and the taxonomic groups (Fig.36), the results obtained are I= 0.467 and Chi-square p= 0.000, is regarded as a strong associative and statistically significant analysis of clusters relationships. The new dimensions allow us to evidence the associations between the clusters: 1-4, 1-1, 1-2, 2-1, and 3-3 (in all the cases, clusters are written in the order qualitative and quantitative typology). Once again, the 4 cluster of the taxonomic groups are isolated from the others in the fourth quadrant.

This means that the quantitative variables did not help to separate the *R. sanguineus* and *Intermediate* clusters, evidencing again the big intra variation present within this species. The control cluster (*R. pusillus* – 4 taxonomic cluster) helps to validate the analysis, being isolated from the others. Only the *R. turanicus* cluster was clearly in association with one quantitative variables cluster, the 3, helping to clarify the different morphological variation between this and the *R. sanguineus* group. The cluster 3 has specimens with average size conscutum, short palps, medium basis capituli and adanal plates, and small spiracles with medium angles and with large final width of the tail (as large as the adjacent festoon). These features are in concordance with some descriptions for *R. turanicus* morphology.

4.3. Latest Ultramorphologic and Morphological Classification

Due to the wide variety highlighted by the statistical analysis, especially within the clusters associated with the "*R. sanguineus"*, we proceeded with another taxonomic analysis on SEM and LS photos (and also accessing all the data obtained from morphological and statistical analysis) with the purpose to unfold it. To do so we based on the latest classification suggested by Dantas-Torres et al. (2013). This classification takes already into consideration the great intraspecific variability within the *R. sanguineus* group, and may be more enlightening than the traditional taxonomic analysis.

The whole variability observed in the males' *R. sanguineus* group can be resumed to the general description that follows:

- ***Body:*** oval shaped, from yellowish brown to reddish brown in colour;
- ***Capitulum:*** wider than long; basis capituli with acute lateral angles; short palps, tapering until the vertex and rounded apically;

- ***Conscutum:*** longer than wider, narrower anteriorly; eyes almost flat surrounded by a few large or setiferous punctations; deep and in comma shape cervical pits, and posteriorly convergent; deep marginal lines with more or less punctuations, sometimes with setiferous punctuation, posteriorly extend to the second festoon and ending anteriorly behind the eyes; elongated posteromedian groove and paramedian grooves with variable length and shape (such as circular, oval, and comma shape continuous with the adjacent festoon limit); usually with an evident "*simus"* pattern (four longitudinal rows of large and deep setiferous punctations, running from the eyes level into the posterior grooves); interstitial punctation variable in size and density;
- ***Spiracular plate:*** variable plate morphology, from a long comma shape with a tail ending with less than half of the adjacent festoon width to a short and almost oval shape, with a tail ending equal to half or with the same adjacent festoon width;
- ***Adanal plates:*** longer than broad, and variable in shape.

The observed and described variability can then be unfolded in several groups that share similar morphological traits. Four of them were formed based on the description given by Dantas-Torres et al. (2013), but others did not feat on their descriptions. Based on that, some new identification references were chosen: *R. sanguineus* type a., *R. sanguineus* type b., *R. turanicus* type a., and *R. turanicus* type b.

Thus, and based on the conscutum size, adanal plates, spiracular plate shape, and tail ending, the following morphologies were identified among the males studied specimens:

- ***R. sanguineus* African type** – based on Dantas-Torres et al. (2013) *R. sanguineus s.l.* description, with longer and narrower conscutum, longer and thinner spiracle plates, very thin ending tail (inferior to half of the adjacent festoon width), higher adanal plates. See Fig.37 and Fig.38.
- ***R. sanguineus* type I** – based on Dantas-Torres et al. (2013) *Rhipicephalus* sp. I description, with longer and narrow conscutum, a quite shorter spiracular plate, a very thin tail ending, higher adanal plates with internal angles slightly rounded and robust accessory plates than the African type. See Fig.39 and Fig.40.
- ***R. sanguineus* type II** – based on Dantas-Torres et al. (2013) *Rhipicephalus* sp. II description, with longer and narrow conscutum, slightly shorter spiracular plate with a

thinner tail ending, adanal plates with the internal angle projected behind and the postero-external angle rounded, and thinner accessory plates than type I. See Fig.41 and Fig.42.

- ***R. sanguineus* type a**. – differ from the other types' description, with longer and narrow conscutum, long and thinner spiracular plate, adanal plates rounded with internal angle projected behind. See Fig.43 and Fig.44.
- ***R. sanguineus* type b**. – differ from the other types description, with a more globular spiracular body, but a thinner tail ending, with adanal plates rounded with internal angle projected behind. See Fig.45.
- ***R. turanicus* –** based on Papadopoulos et al. (1992), Walker et al. (2003), and Dantas-Torres et al. (2013) descriptions; characterized by an average sized conscutum, shorter palps, medium sized basis capituli and adanal plates, shorter spiracular plates with medium angles and large tail ending width (larger than half of the adjacent festoon width, sometimes as larger). See Fig.46 and Fig.47.
- ***R. turanicus* type a**. – differ from the other types descriptions, it shows similar features to *R. turanicus*, although spiracular plate has a thinner tail ending (inferior to half of the adjacent festoon width). See Fig.48 and Fig.49.
- ***R. turanicus* type b**. – differ from the other types descriptions, it shows similar features to *R. turanicus*, but shorter and oval spiracular plate, with a very short and large tail ending. See Fig.50 and Fig.51.

For better viewing, the figures are shown in the next pages (from pg.83 to pg.91).

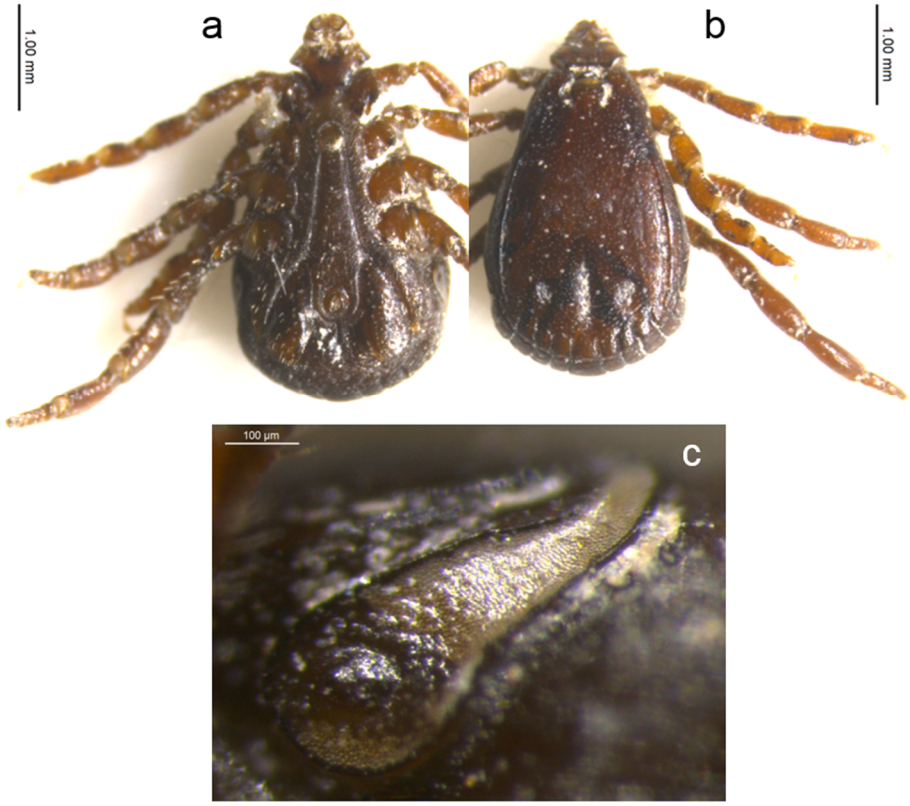

Fig.37 – Male tick, ID=CZ CR1534 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* African type. (a) body ventral view: higher adanal plates; (b) body dorsal view: longer and narrower conscutum; (c) spiracular plate: longer and thinner, thin tail ending (inferior to half of the adjacent festoon width).

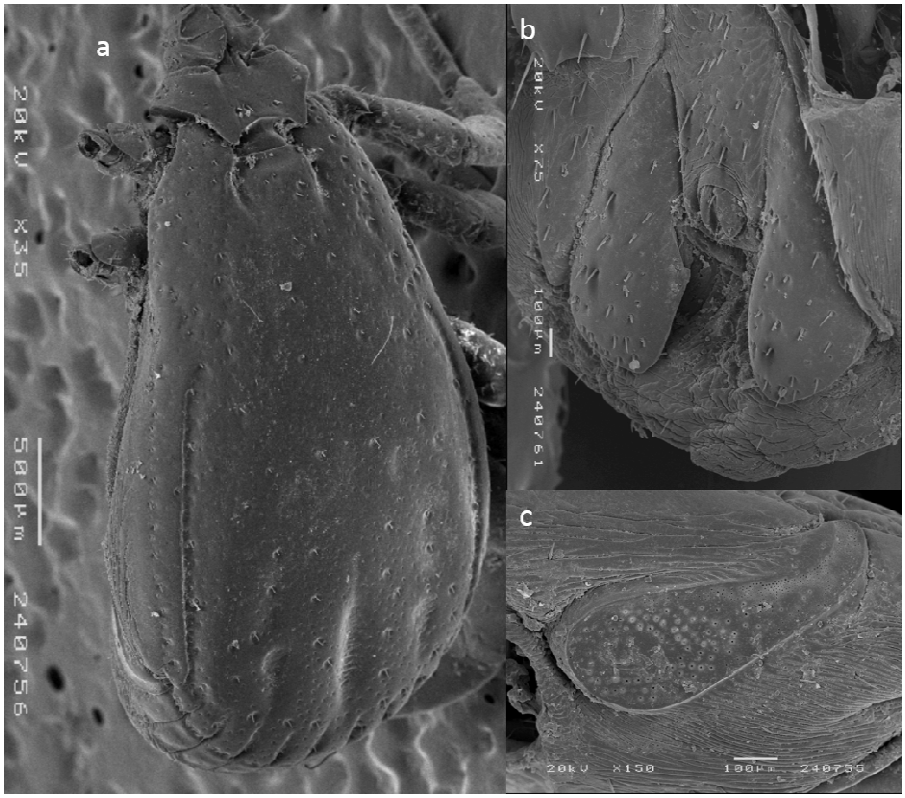

Fig.38 – Male tick, ID=CZ S1255 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* African type. (a) body dorsal view: longer and narrower conscutum; (b) adanal plates: higher and robust; (c) spiracular plate: longer and thinner, thin tail ending (inferior to half of the adjacent festoon width).

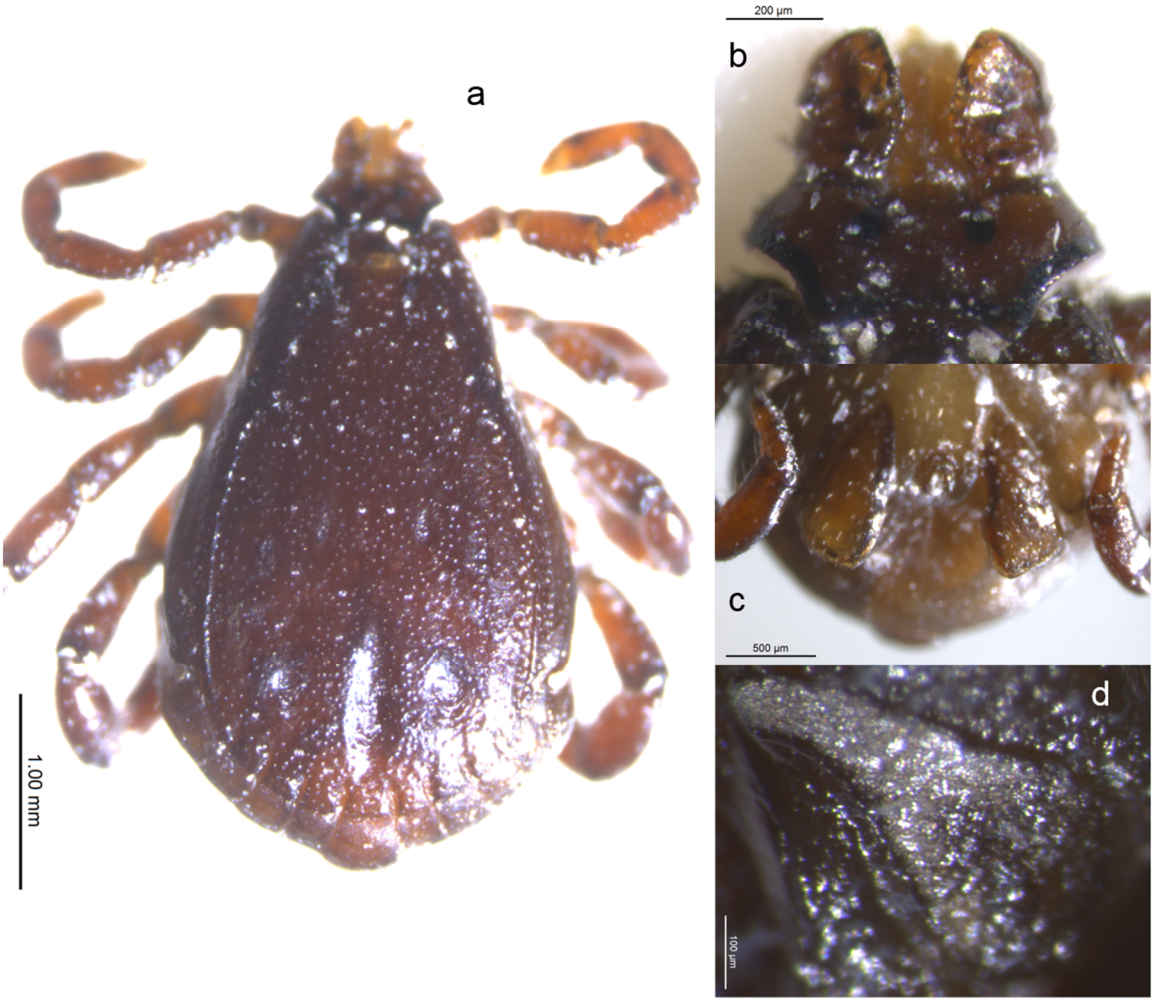

Fig.39 – Male tick, ID=CZ O181 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type I. (a) body dorsal view: longer and narrow conscutum; (b) dorsal capitulum view; (c) adanal plates: high, with internal angles slightly rounded and more robust accessory plates than the African type; (d) spiracular plate: shorter, thin tail ending.

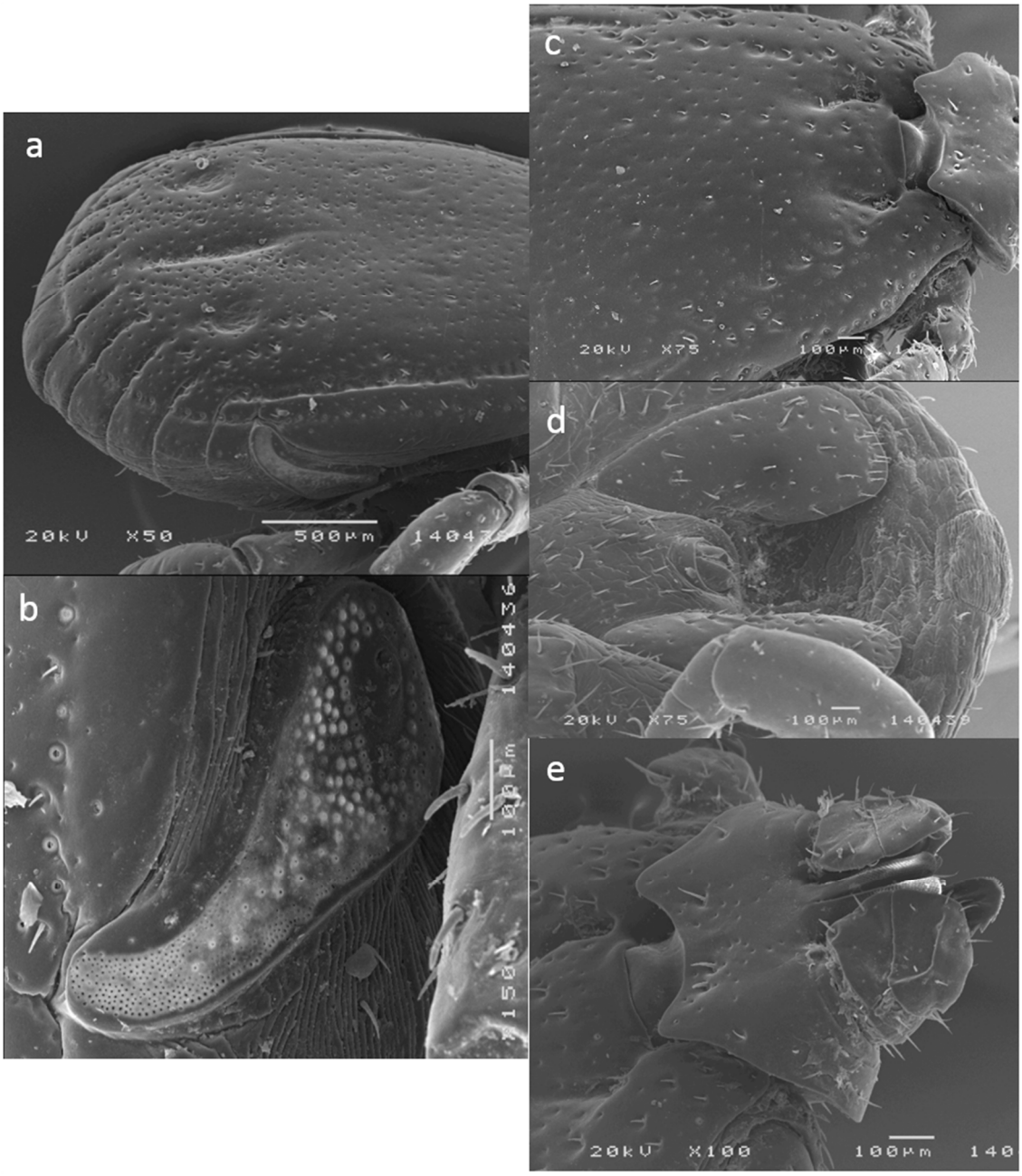

Fig.40 – Male tick, ID=CZ ExS1 (IICT). Evaluation according to Dantas-Torres (2013): *R. sanguineus* type I. This specimen was not part of the data set analysed, but as its morphology fits the group description, one display above these SEM photos as an example of the ones that had been analysed. (a) Posterior margin of conscutum: longer and narrow; (b) spiracular plate: short, with thin tail ending; (c) anterior margin of conscutum, long and narrow; (d) adanal plates: high, with internal angles slightly rounded and robust accessory plates than the African type; (e) dorsal view of capitulum.

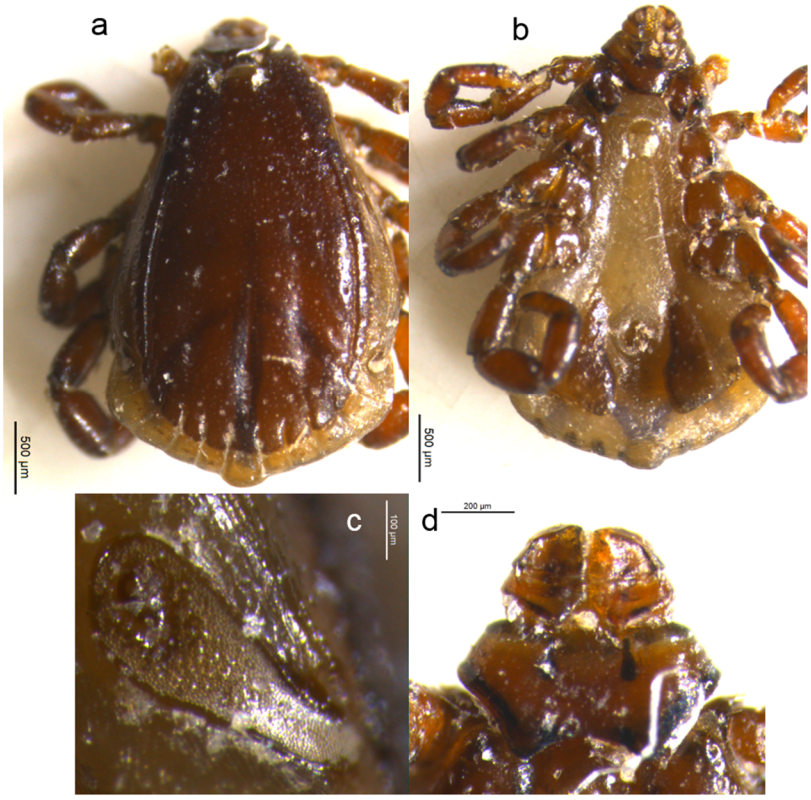

Fig.41 – Male tick, ID=CZ CR1562 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type II. (a) body dorsal view: longer and narrow conscutum; (b) body ventral view: adanal plates with the internal angle projected behind and the postero-external angle rounded, and thinner accessory plates than type I; (c) spiracular plate: short spiracular plate with a thinner tail ending; (d) dorsal view of the capitulum.

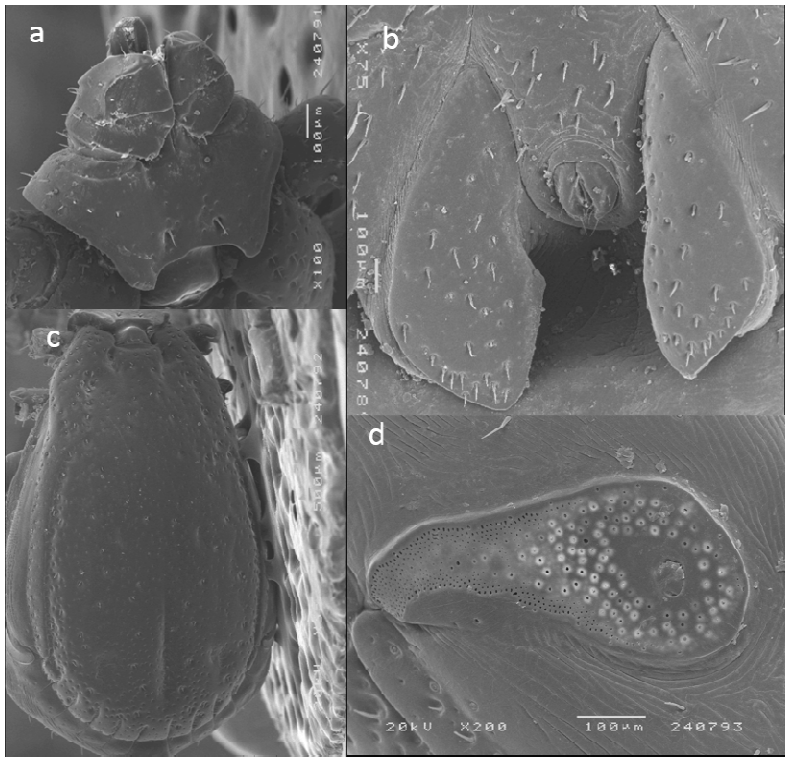

Fig.42 – Male tick, ID=CZ S1328 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type II**.** Present a longer and narrow conscutum, slightly shorter spiracular plate with a thinner tail ending, adanal plates with the internal angle projected behind and the postero-external angle rounded, and thinner accessory plates than type I. (a) capitulum dorsal view; (b) adanal plates; (c) conscutum; (d) adanal plate.

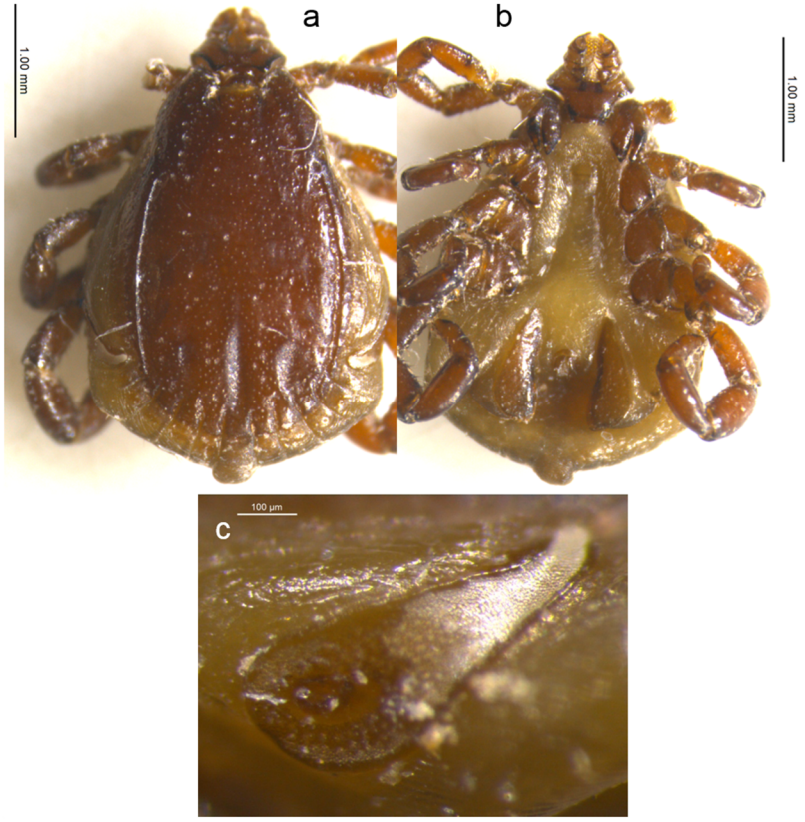

Fig.43 – Male tick, ID=CZ CR1549 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type a**.** It differ from the other types' description by presenting a longer and narrow conscutum, long and thinner spiracular plate, adanal plates rounded with internal angle projected behind. (a) Body dorsal view; (b) body ventral view; (c) spiracular plate.

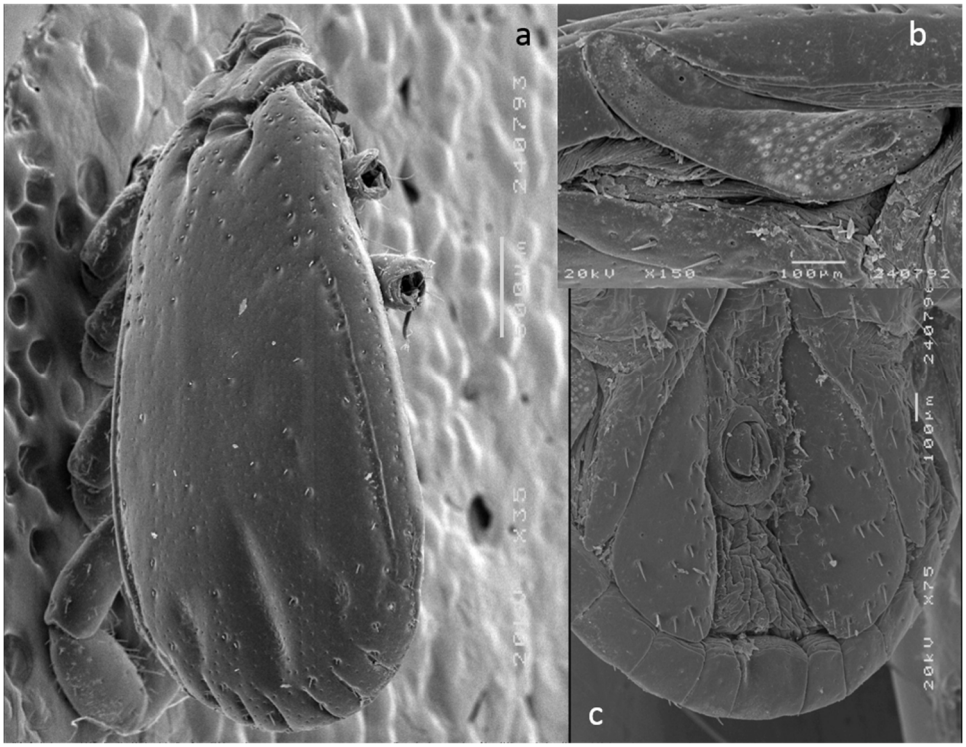

Fig.44 – Male tick, ID=CZ O136 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type a**.** It differ from the other types' description, by presenting ah longer and narrow conscutum, long and thinner spiracular plate, adanal plates rounded with internal angle projected behind**.** (a) Dorsal body view; (b) spiracular plate; (c) adanal plates.

Fig.45 – Male tick, ID=CZ CR1571 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type b**.** It differ from the other types description by presenting a more globular spiracular body, but with a thinner tail ending, and adanal plates rounded with internal angle projected behind. (a) Body dorsal view; (b) body ventral view; (c) spiracular plate.

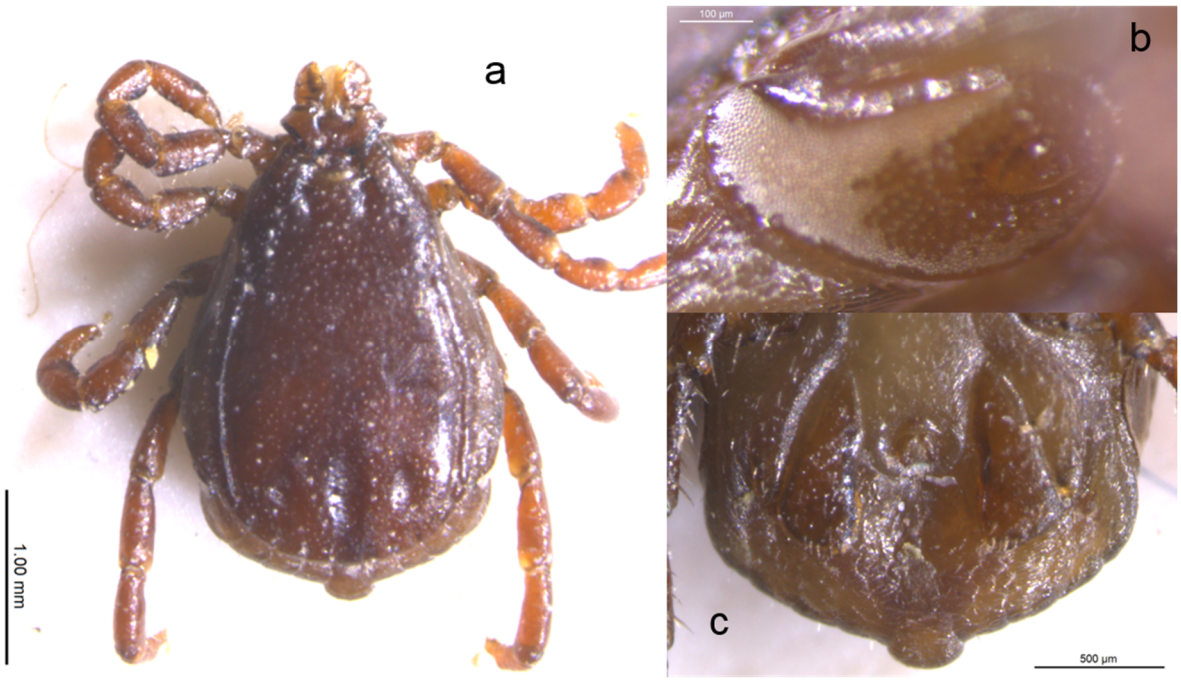

Fig.46 – Male tick, ID=CZ O129 (IICT). Evaluation according Dantas-Torres (2013), Papadopoulos et al. (1992), and Walker et al. (2003): *R. turanicus.* It characterized by an average sized conscutum, shorter palps, medium sized basis capituli and adanal plates, shorter spiracular plates with medium angles and large tail ending width (larger than half of the adjacent festoon width, sometimes as larger). (a) Body dorsal view; (b) spiracular plate; (c) adanal plates.

Fig.47 – Male tick, ID=CZ CR1542 (IICT). Evaluation according Dantas-Torres (2013), Papadopoulos et al. (1992), and Walker et al. (2003): *R. turanicus*. It characterized by shorter palps, and medium sized basis capituli and adanal plates, (a) Average sized conscutum; (b) adanal plates; (c) capitulum dorsal view; (d) spiracular plate: short, with medium angles, and large tail ending width (larger than half of the adjacent festoon width, sometimes as larger).

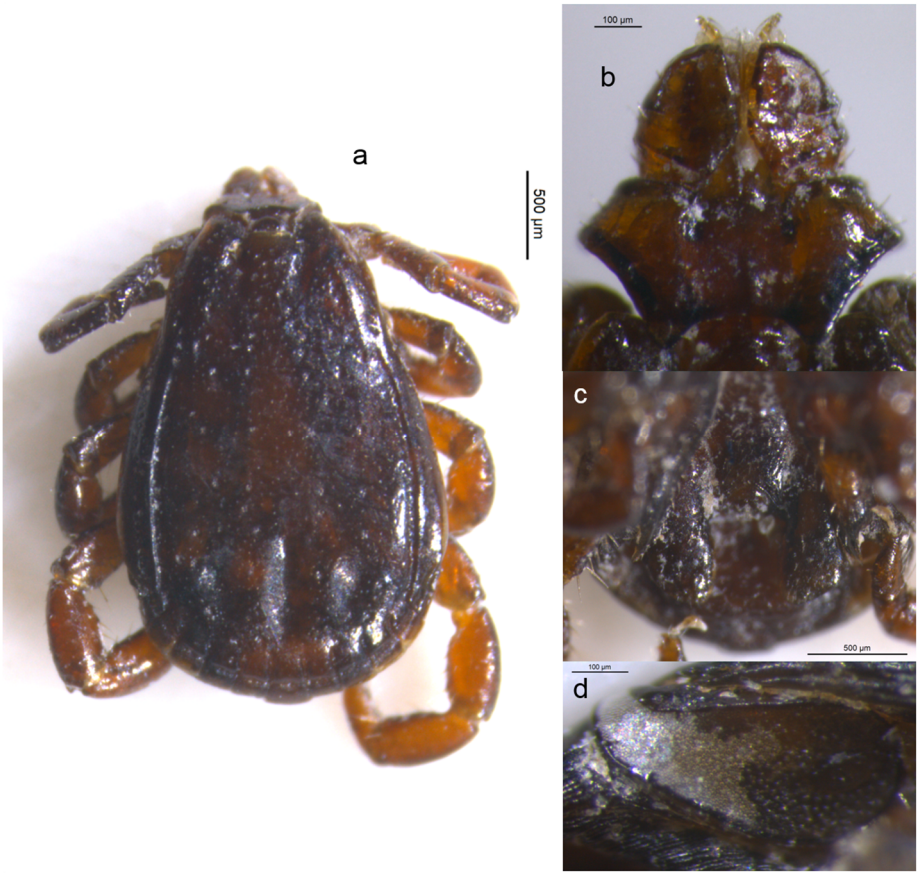

Fig.48 – Male tick, ID=CZ S273 (IICT). Evaluation according Dantas-Torres (2013): *R. turanicus* type a**.** It shows similar features to *R. turanicus*, although its spiracular plate has a thinner tail ending (inferior to half of the adjacent festoon width). (a) Body dorsal view; (b) spiracular plate; (c) adanal plates.

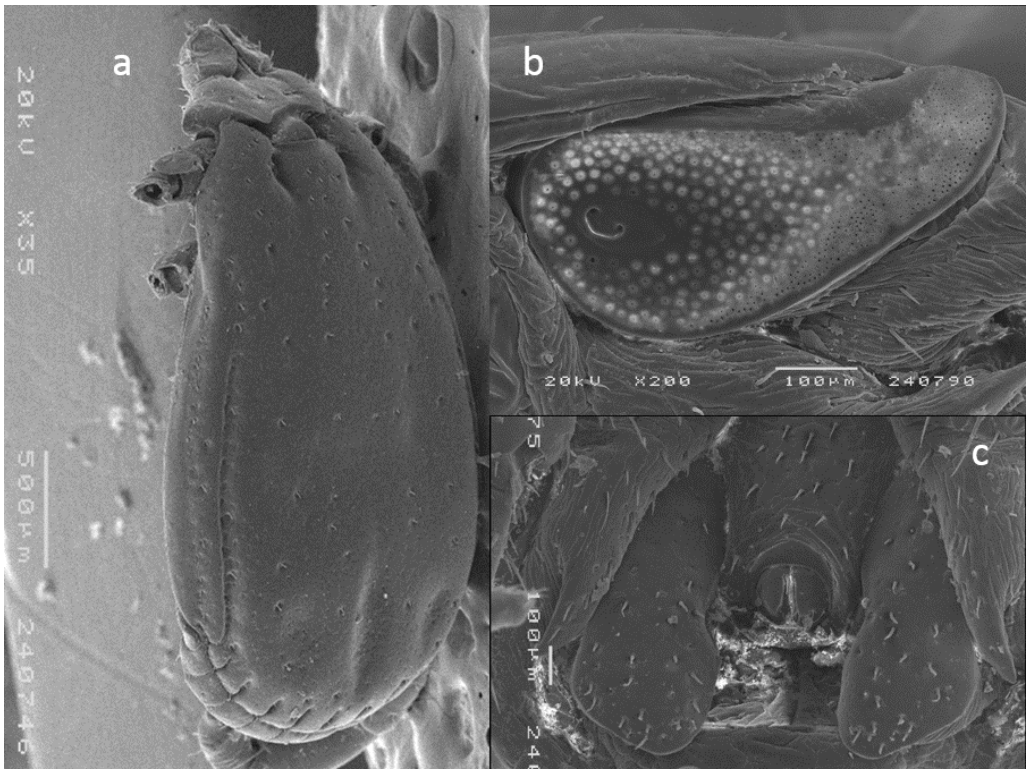

Fig.49 – Male tick, ID=CZ S273 (IICT). Evaluation according Dantas-Torres (2013):*R. turanicus* type a**.** It shows similar features to *R. turanicus*, although its spiracular plate has a thinner tail ending (inferior to half of the adjacent festoon width). (a) dorsal body view; (b) spiracular plate; (c) adanal plates.

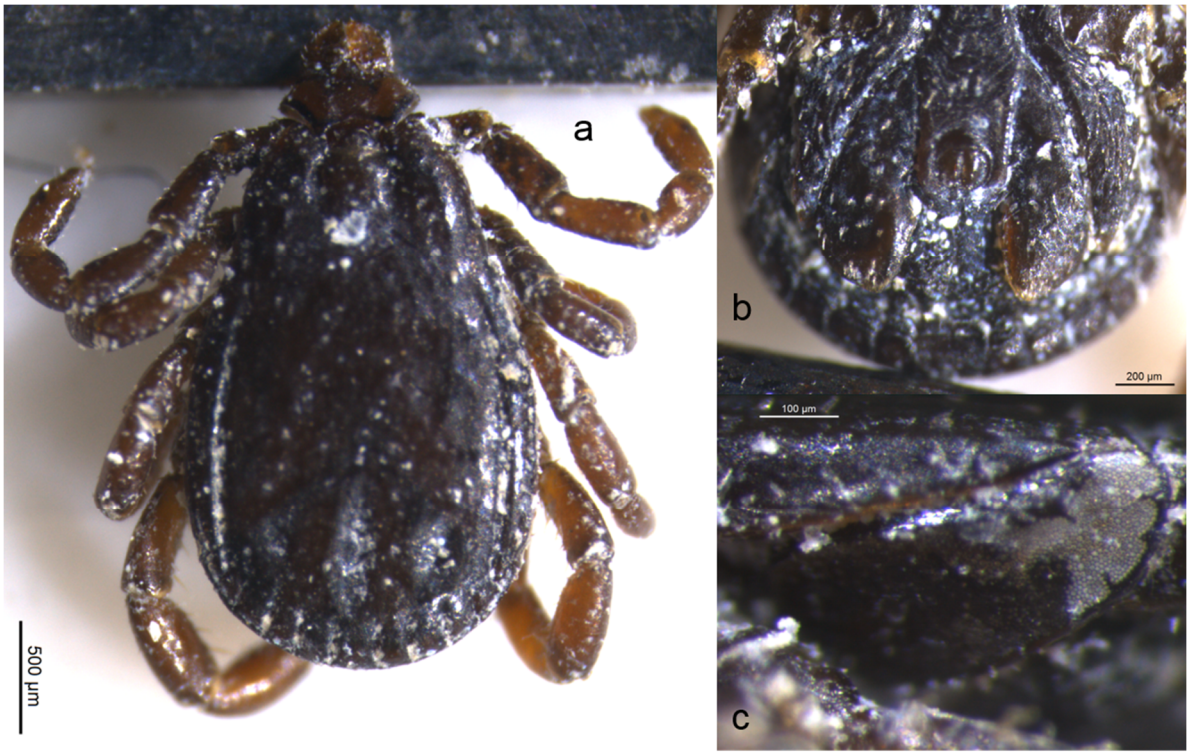

Fig.50 – Male tick, ID=CZ S449 (IICT). Evaluation according Dantas-Torres (2013):*R. turanicus* type b**.** It shows similar features to *R. turanicus*, but presented a shorter and oval spiracular plate, with a very short and large tail ending. (a) Body dorsal view; (b) adanal plates; (c) spiracular plate.

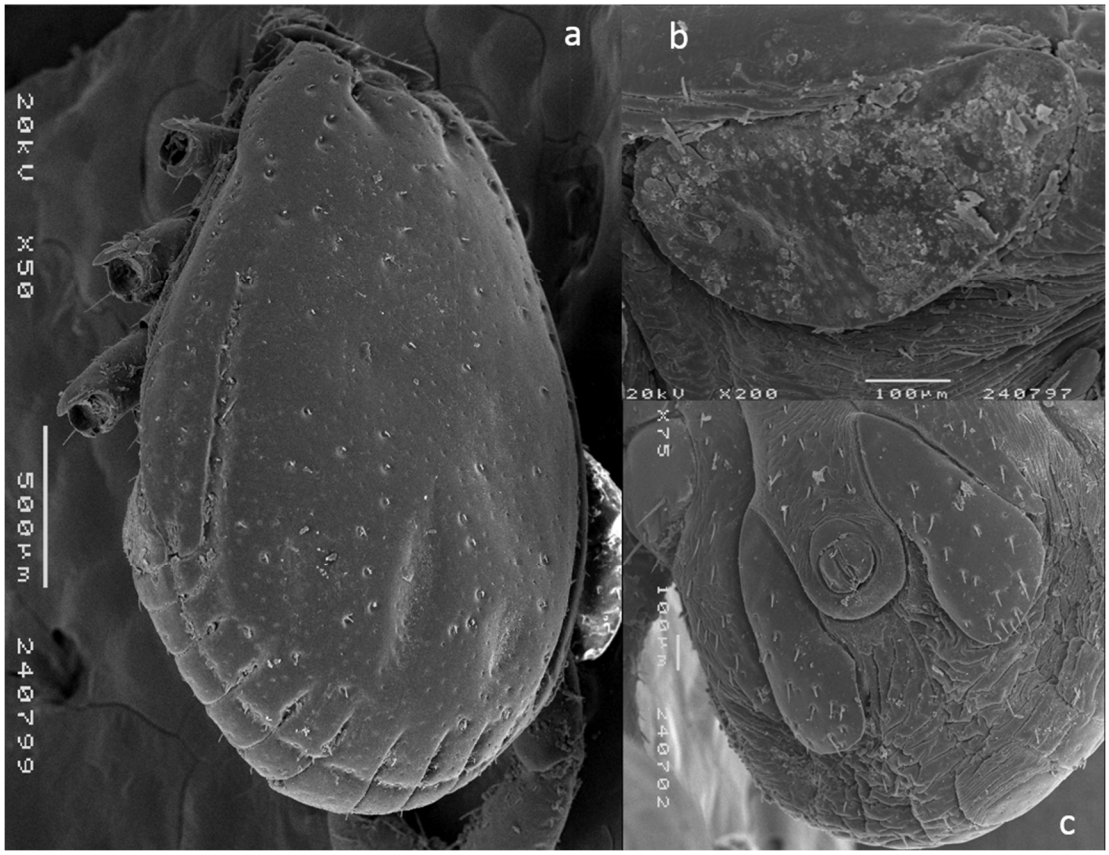

Fig.51 – Male tick, ID=CZ CR1527 (IICT). Evaluation according Dantas-Torres (2013):*R. turanicus* type b**.** It shows similar features to *R. turanicus*, but presented a shorter and oval spiracular plate, with a very short and large tail ending. (a) Conscutum; (b) spiracular plate; (c) adanal plates.

Referring now the females, the whole variability observed in this gender *R. sanguineus* group can be resumed to the general description follow:

- ***Body:*** oval shaped, from yellowish brown to reddish brown in colour;
- ***Capitulum:*** wider than long; posterior porose areas in the basis capituli; small porose areas, from circular to slightly oval shape; tapering palps to rounded vertexes;
- ***Scutum:*** longer than wide; sinuous posterior margin; punctation deepness and distribution variable, and seems more dense comparatively to males; outlined lateral grooves with large punctations; clearly defined cervical fields depression, densely punctuated, external cervical margins marked by several larger punctations or setiferous punctations; few larger punctuations present in the scapular areas.
- ***Genital aperture:*** U shaped with a narrow or wider;
- ***Spiracular plates****:* from round-shaped to oval-shaped, with a shorter tail than in males.

The observed and described variability can then be unfolded in several groups that share similar morphological traits. Two of them were formed based on the description given by Dantas-Torres et al. (2013), but others did not feat on their descriptions. Based on that, some new identification references were chosen: *R. sanguineus* type A., *R. sanguineus* type B., *R. sanguineus* type C., *R. turanicus*, and *R. turanicus* type A. It is to note that these names are just for facilitate the reference to the morphological group, and do not have to pair with the males with the same reference.

Thus, and based on conscutum size, genital aperture, scutum posterior margin, and spiracular plate shape, the following morphologies were identified among the females studied specimens:

- ***R. sanguineus*** **African type** – based on Dantas-Torres et al. (2013) *R. sanguineus s.l.* description. See Fig.52 and Fig.53.
- ***R. sanguineus*** **type II –** based on Dantas-Torres et al. (2013) *Rhipicephalus* sp. II description. See Fig.54 and Fig.55. It is the most frequent morphology in our study group.
- ***R. sanguineus*** **type A. –** differ from the other types descriptions, due to slightly oval-shaped spiracular plate with a very short tail. See Fig.56.

- ***R. sanguineus* type B. –** differ from the other types descriptions, because it presents a large, oval-shaped spiracular plate with a very long and curved tail; genital aperture operculum large and long. See Fig.57 and Fig.58.
- ***R. sanguineus* type C.** – differ in the shorter spiracular plate body, genital aperture with long sclerites projected backwards the aperture posterior border. See Fig.59.
- ***R. turanicus*** – differs from the Italian *R. turanicus* specimen (Italian specimen kindly provided by Dantas-Torres to comparison – data not shown) by higher sclerites of genital aperture and slightly smaller spiracular plate. See Fig.60 and Fig.61. This is a more frequent morphology within the *R. turanicus* group.
- ***R. turanicus* type A**. – It resembles the Italian *R. turanicus* specimen (data not shown) by similar sclerites of genital aperture, but it differs in the smaller spiracular plate. See Fig.62.

For better viewing, the figures are shown in the next pages (from this page to pg.99).

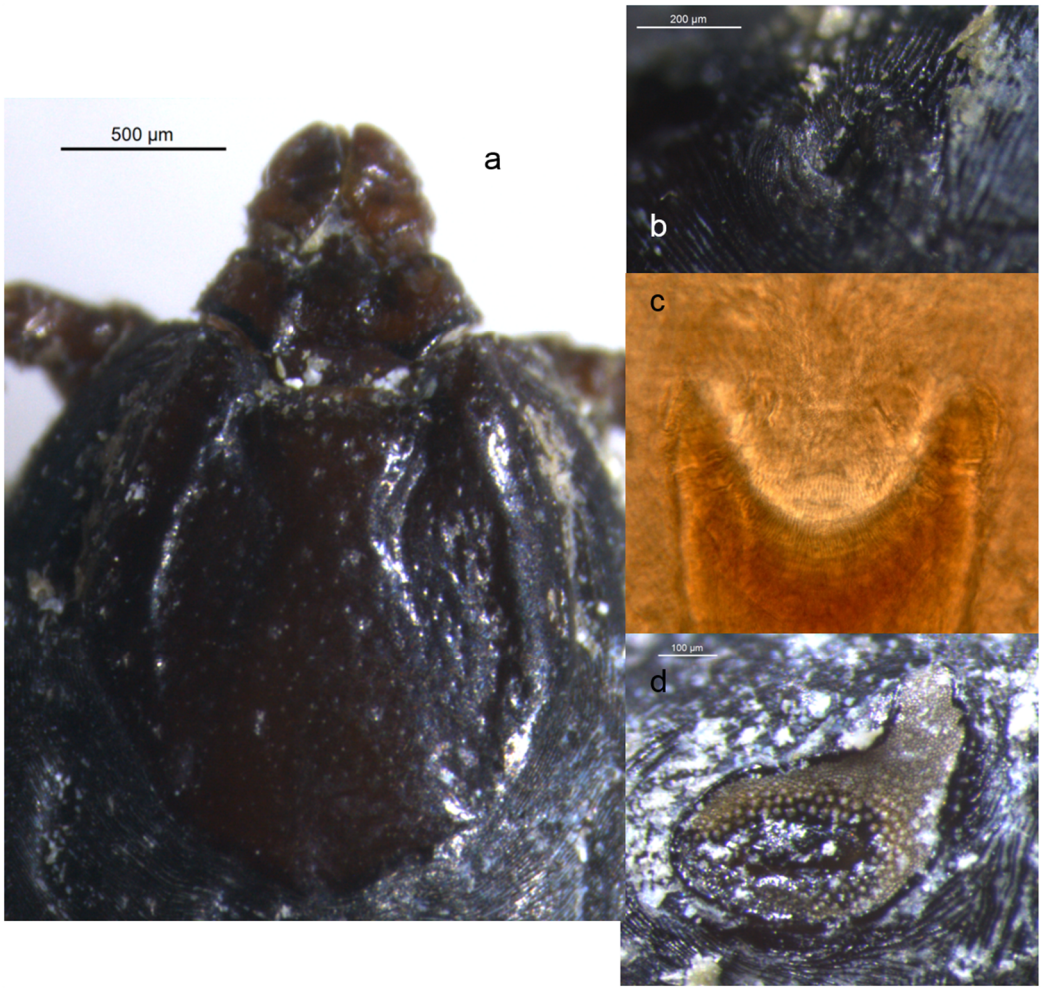

Fig.52 – Female tick, ID=CZ S840 (IICT). Evaluation according Dantas-Torres (2013):*R. sanguineus* African type. (a) scutum and capitulum; (b) genital aperture; (c) genital aperture mounted; (d) spiracular plate.

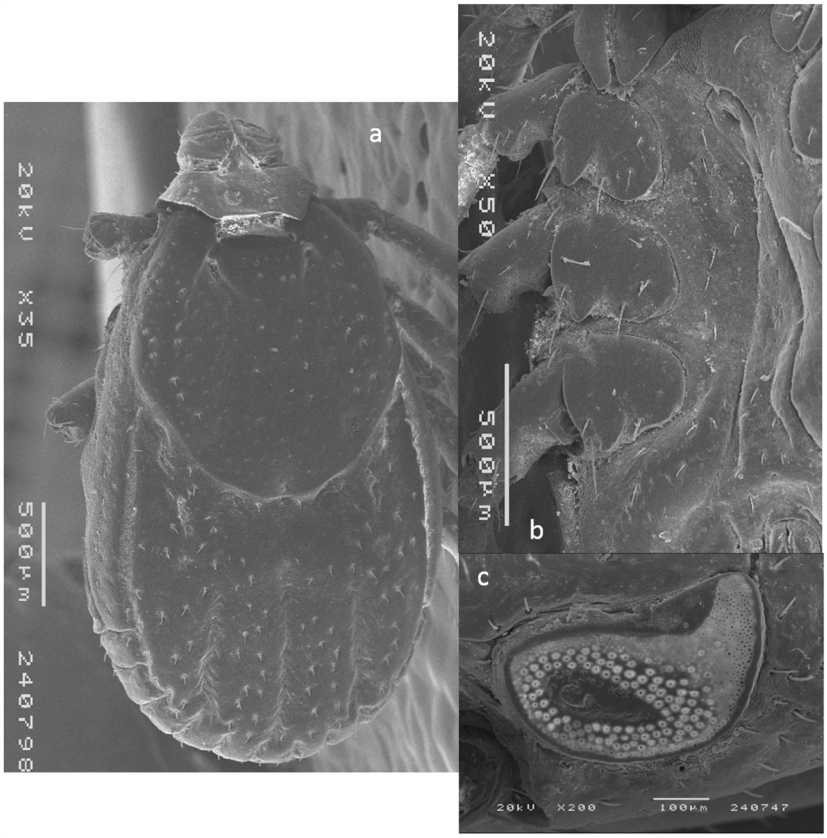

Fig.53 – Female tick, ID=CZ O216 (IICT). Evaluation according Dantas-Torres (2013):*R. sanguineus* African type. (a) body dorsal view; (b) genital aperture; (c) spiracular plate.

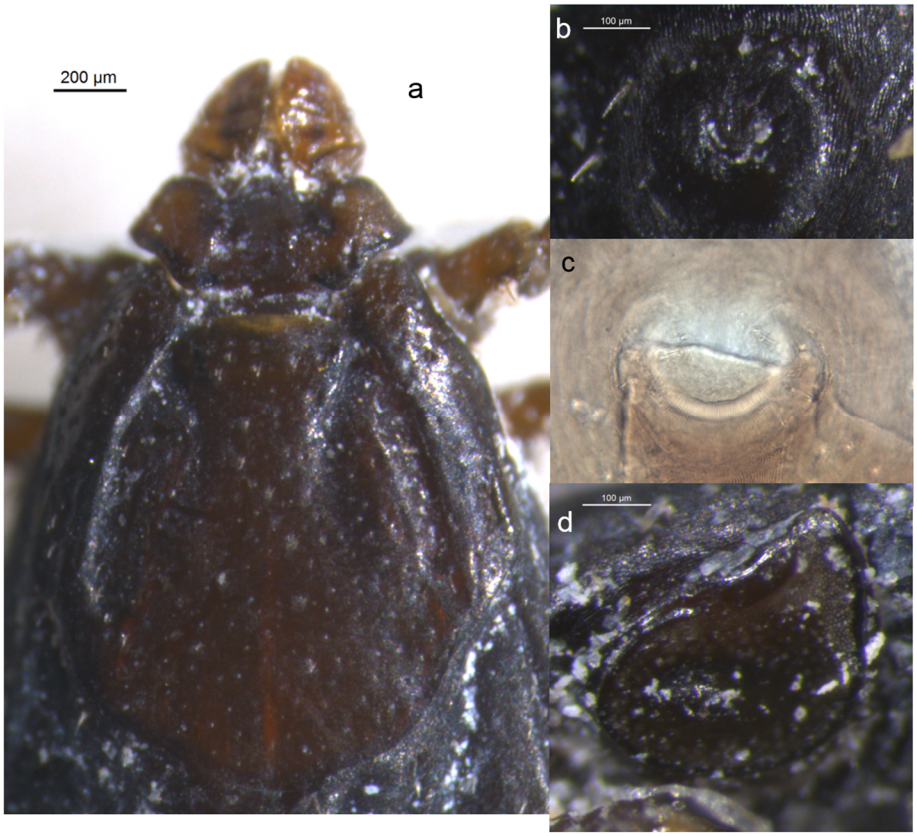

Fig.54 – Female tick, ID=CZ S563 (IICT). Evaluation according Dantas-Torres (2013):*R. sanguineus* type II. It is the most frequent morphology in our females study group**.** (a) scutum and capitulum; (b) genital aperture; (c) mounted genital aperture; (d) spiracular plate.

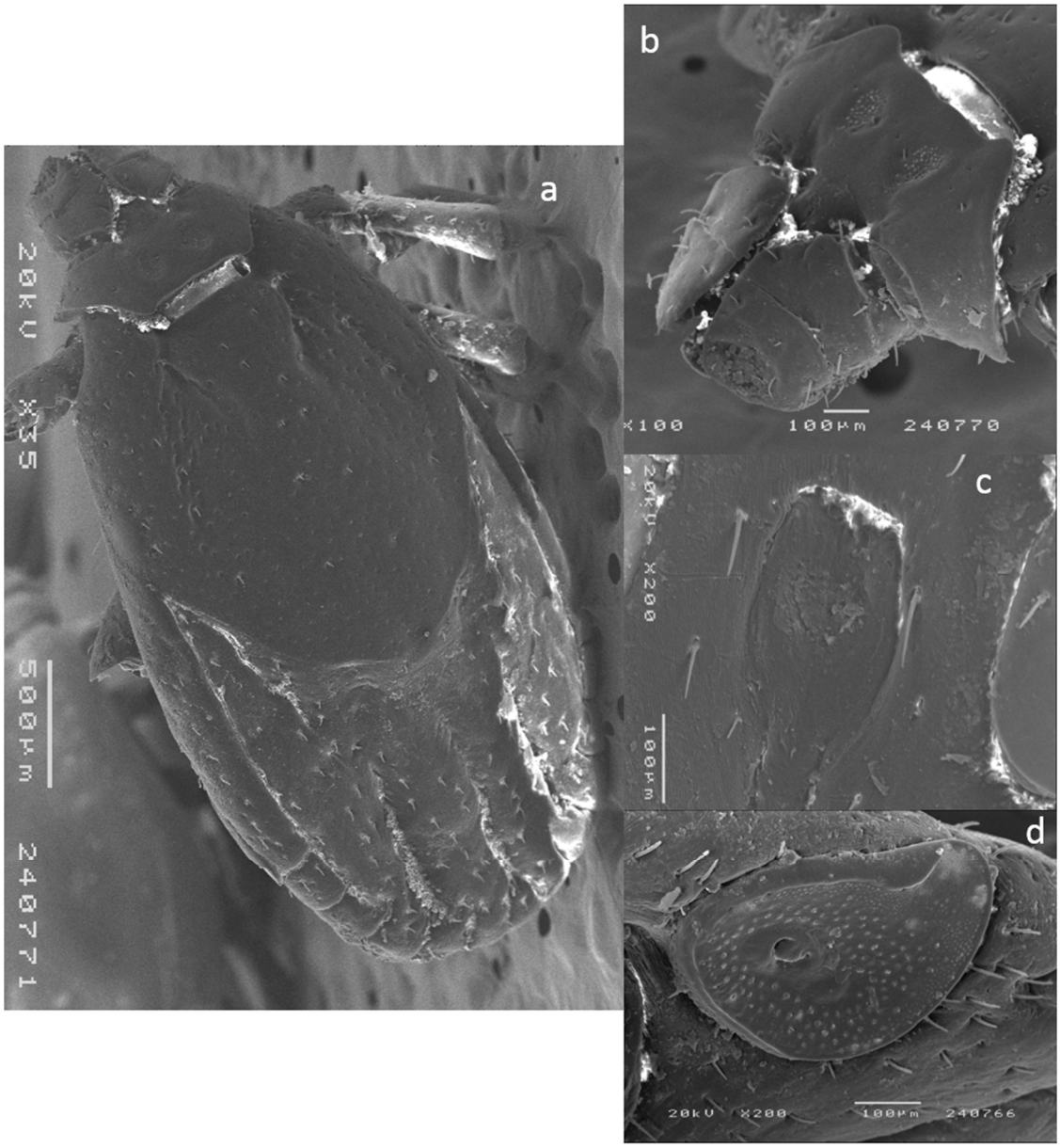

Fig.55 – Female tick, ID=CZ S466 (IICT). Evaluation according Dantas-Torres (2013):*R. sanguineus* type II. It is the most frequent morphology in our females study group. (a) body dorsal view; (b) capitulum dorsal view; (c) genital aperture; (d) spiracular plate.

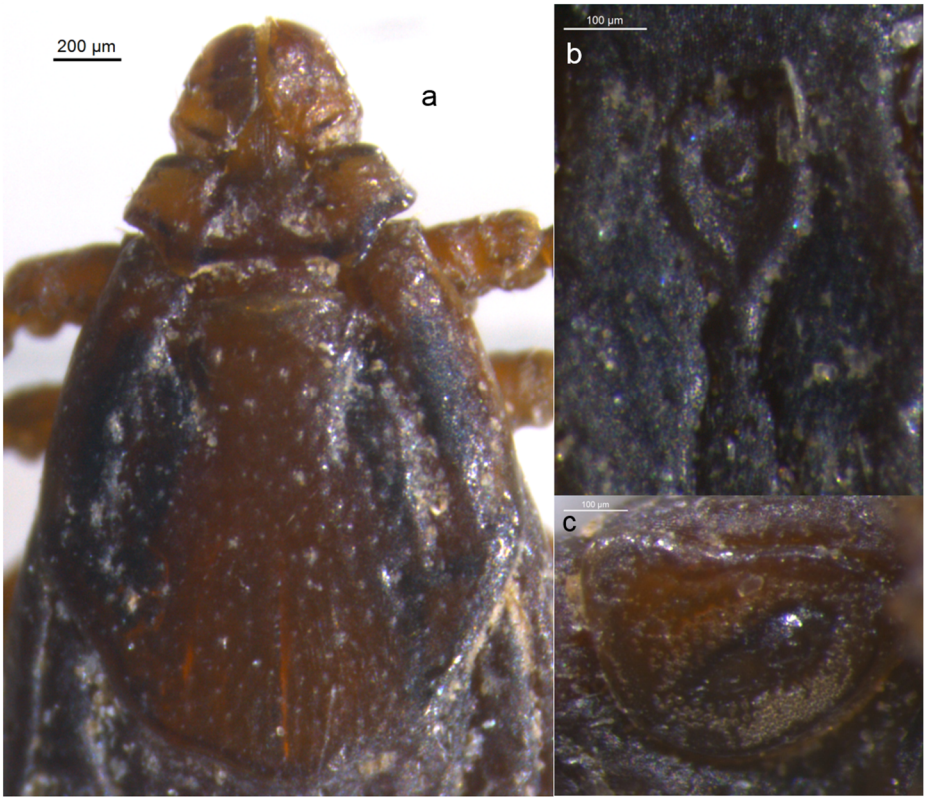

Fig.56 – Female tick, ID=CZ S1090 (IICT). Evaluation according Dantas-Torres (2013):*R. sanguineus* type A. It differ from the other types descriptions due to the slightly oval-shaped spiracular plate with a very short tail. (a) scutum; (b) genital aperture; (c) spiracular plate.

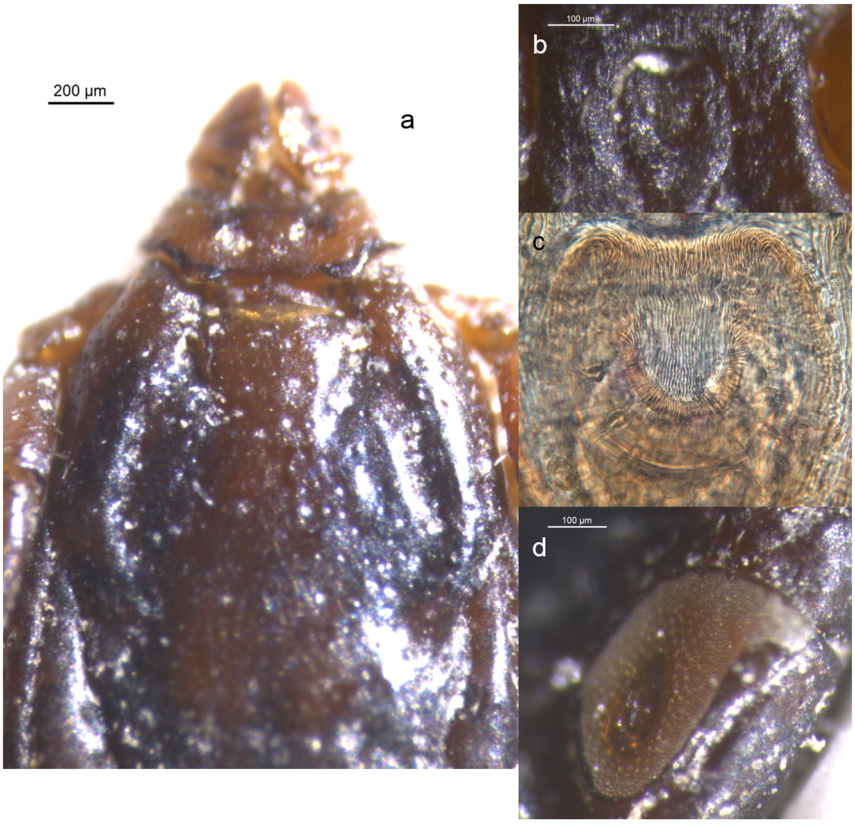

Fig.57 – Female tick, ID=CZ O1346 (IICT). Evaluation according Dantas-Torres (2013):*R. sanguineus* type B. It differ from the other types descriptions by presenting a large, oval-shaped spiracular plate with a very long and curved tail; and a genital aperture operculum large and long**.** (a) scutum and capitulum; (b) genital aperture; (c) mounted genital aperture; (d) spiracular plate.

Fig.58 – Female tick, ID=CZ O1347 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type B. It differ from the other types descriptions by presenting a large, oval-shaped spiracular plate with a very long and curved tail; and a genital aperture operculum large and long**.** (a) scutum and capitulum; (b) genital aperture; (c) spiracular plate.

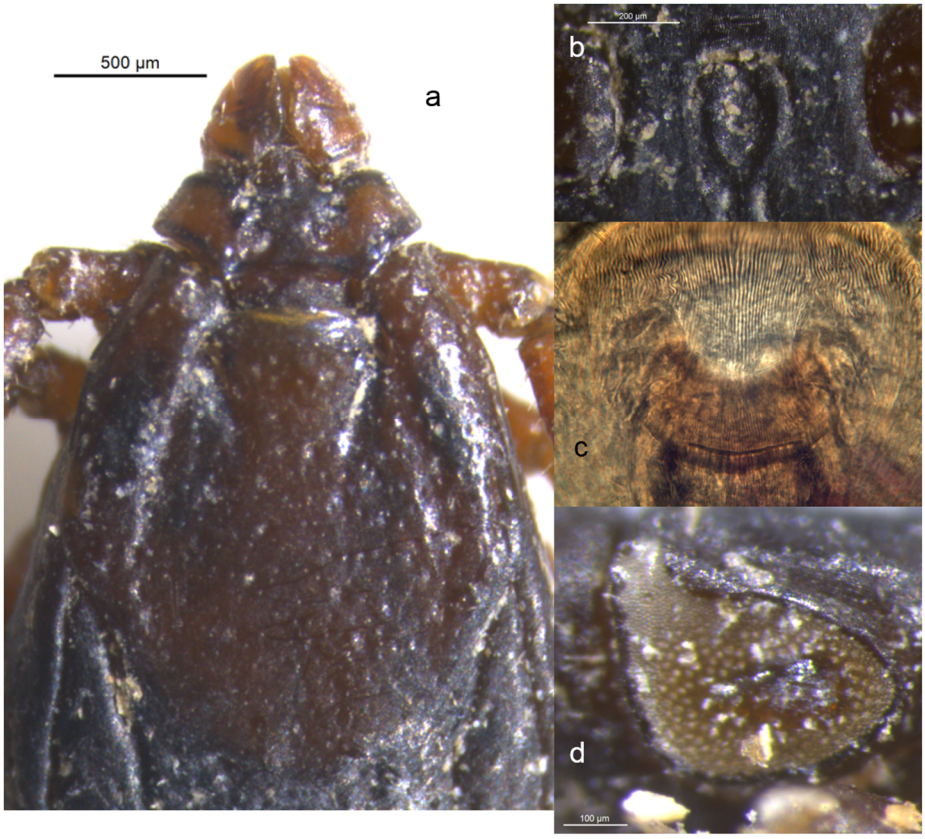

Fig.59 – Female tick, ID=CZ S1282 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type C. (a) scutum and capitulum; (b) genital aperture; (c) mounted genital aperture: with long sclerites projected backwards the aperture posterior border; (d) spiracular plate: short spiracular plate body.

Fig.60 – Female tick, ID=CZ CR1529 (IICT). Evaluation according Dantas-Torres (2013): *R. turanicus*. It differs from the Italian *R. turanicus* specimen (data not shown) by present genital aperture higher sclerites and slightly smaller spiracular plate. This is the more frequent morphology within the *R. turanicus* group. (a) Body dorsal view; (b) genital aperture; (c) mounted genital aperture; (d) spiracular plate.

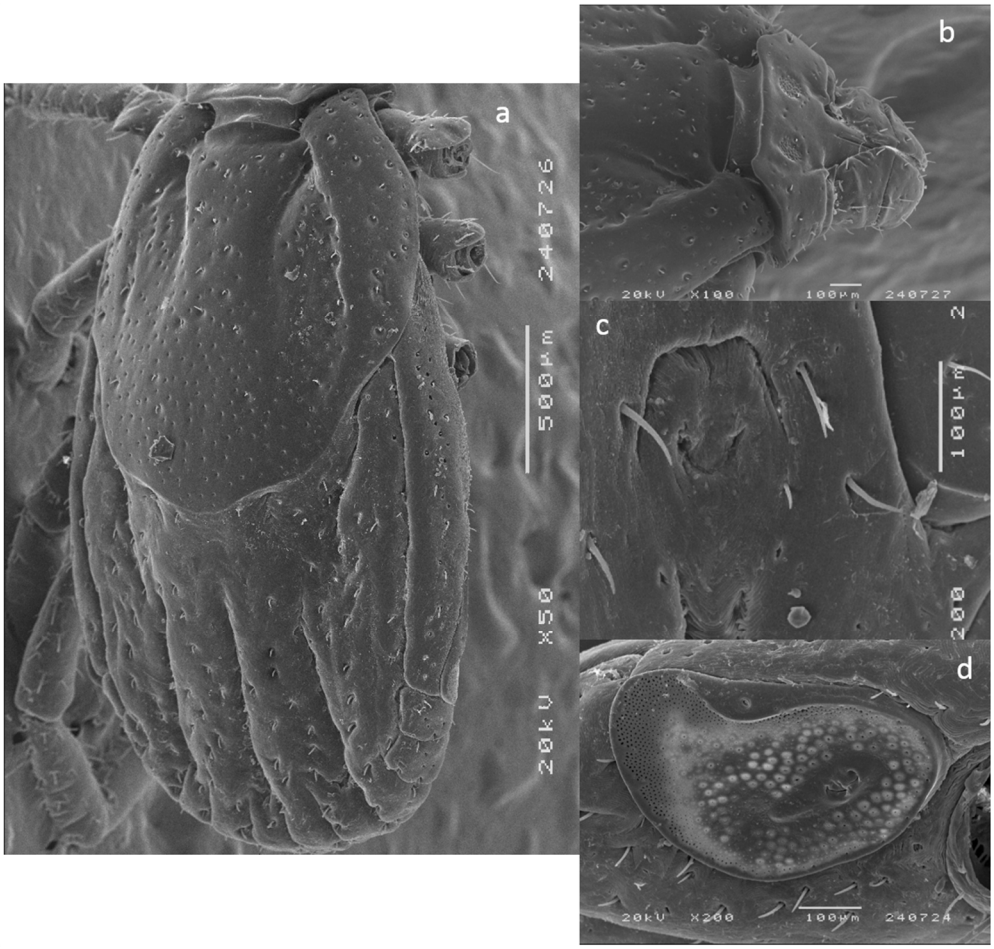

Fig.61 – Female tick, ID= CZ CR1528 (IICT). Evaluation according Dantas-Torres (2013):*R. turanicus***.** It differs from the Italian *R. turanicus* specimen (data not shown) by having genital aperture higher sclerites and slightly smaller spiracular plate. This is the more frequent morphology within the *R. turanicus* group. (a) Scutum and alloscutum; (b) dorsal view of capitulum; (c) genital aperture; (d) spiracular plate.

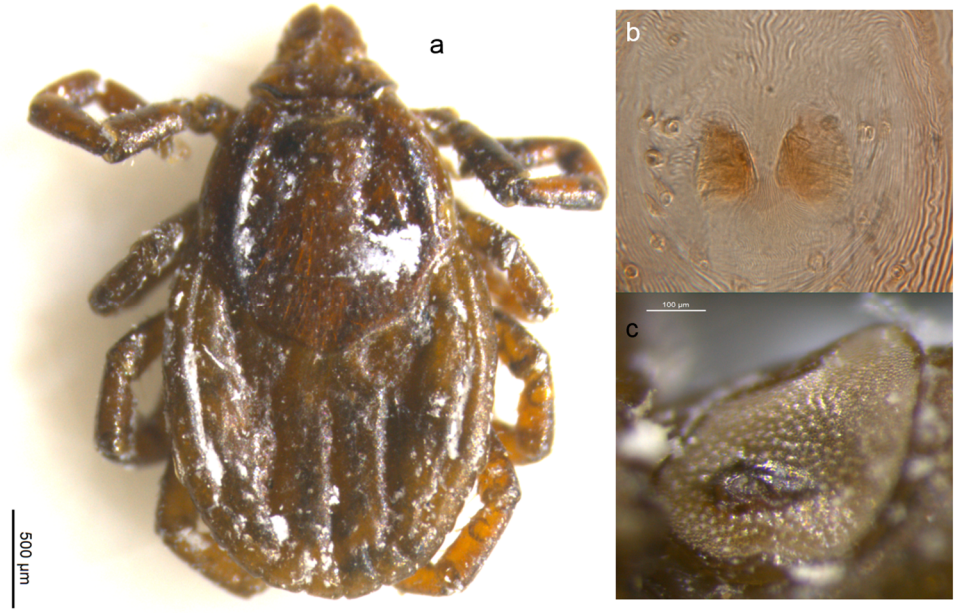

Fig.62 – Female tick, ID= CZ CR1544 (IICT). Evaluation according Dantas-Torres (2013): *R. turanicus* type A**.** It resembles the Italian *R. turanicus* specimen by similar sclerites of genital aperture, but differs in the smaller spiracular plate. (a) Body dorsal view; (b) mounted genital aperture; (c) spiracular plate.

4.4. Molecular Identification

Primer pair COI forward (LCO-1490)/COI reverse (HCO-2198) was not efficient in the recovery of COI sequences from tick specimens. Even so, 22 sequences were identified, and subjected to GenBank BLAST tool comparison analysis. These results are shown in Table.10.

Table.10 – Genetic sequences GenBank identification by genetic homology. (Murrell et al., 2000)**.** ID – CZ identification number (IICT); Dist. – district where the specimen was collected; pb – length of the sequence in base pairs; QC – Query cover; E – e-value; Pct. (%) – homology percentage; Ref. – references of the groups which submitted the homologous sequences.

ID	Sex	Dist.	pb	Accession	QC	E	Pct. (%)	Specie gene	Ref
103	M	O	711	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
119	F	O	715	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
161	F	O	713	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
276	M	S	713	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
291	F	S	712	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
331	M	S	714	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
337	M	S	713	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
358	M	S	711	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
475	M	S	713	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
554	F	S	713	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
827	F	S	713	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1060	F	S	713	AF132839.1	96	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1183	M	S	710	AF132839.1	98	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1535	M	CR	715	AF132839.1	98	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1547	M	CR	787	AF132839.1	86	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1553	M	CR	718	AF132839.1	95	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1563	F	CR	716	JK737086.1	98	0	91	*R. turanicus* isolate Xinjiang COI gene, partial cds	Gou,H., Guan,G., Yin,H. and Luo,J., 2012 (directly submitted)
1565	M	CR	714	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1568	F	CR	743	AF132839.1	92	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1570	M	CR	816	AF132839.1	83	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1573	M	CR	711	AF132839.1	97	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000
1575	M	CR	796	AF132839.1	85	0	99	*R. sanguineus* COI gene, partial cds	Murrell et al., 2000

5. DISCUSSION

5.1. Preliminary Morphological and Statistical analysis

The formed groups according to the traditional morphological analysis were four, namely *R. sanguineus, Intermediate*, *R. turanicus* and *R. pusillus*.

When analysing the Fig.19 and Fig.29, it is possible to verify that their elements proportions are not homogeneous. This clearly indicates that these groups will not favour a strict species separation, which could only be possible if the morphological features chosen for the analysis were sufficiently different between species (what cannot be done in this case due to the intra-variation observed). This must be taken into account in some exceptions that can occur in some clusters. It is also clear, in what concerns this analysis, that *R. sanguineus* species is the predominant species in the studied population. This last observation is in concordance with other studies on this populations (Caeiro, 1999; Rosa et al., 2013, 2006; Santos-Silva, 2010; Santos-Silva et al., 2013, 2011, 2006).

- **Females Specimens Clusters**

During the formation of the female groups, it was observed that the cluster 2 of the quantitative variables had only three female elements: CZ O108, CZ O109, and CZ O141 (IDs of ZC/IICT), shown in Fig.63, Fig.64 and Fig.65, respectively. For better viewing, the referred figures are shown between pg.102 and pg.103.

This separation may have occurred for several reasons, such as because of the fact that spiracular areas of these animals have the higher angles (μ=100.375º), what is outlined by the spiracle tail pointed upwards; and also because their scutums present the lowest values of width/length ratios (μ=1.207, σ=0.403) (see Fig.63, Fig.64 and Fig.65). Additionally, and by comparison with the traditional morphological identification keys (Dias, 1994), these specimens can be *R. pusillus*. However, the only specimen initially identified as one was the CZ O141. The others are identified as *R. sanguineus*.

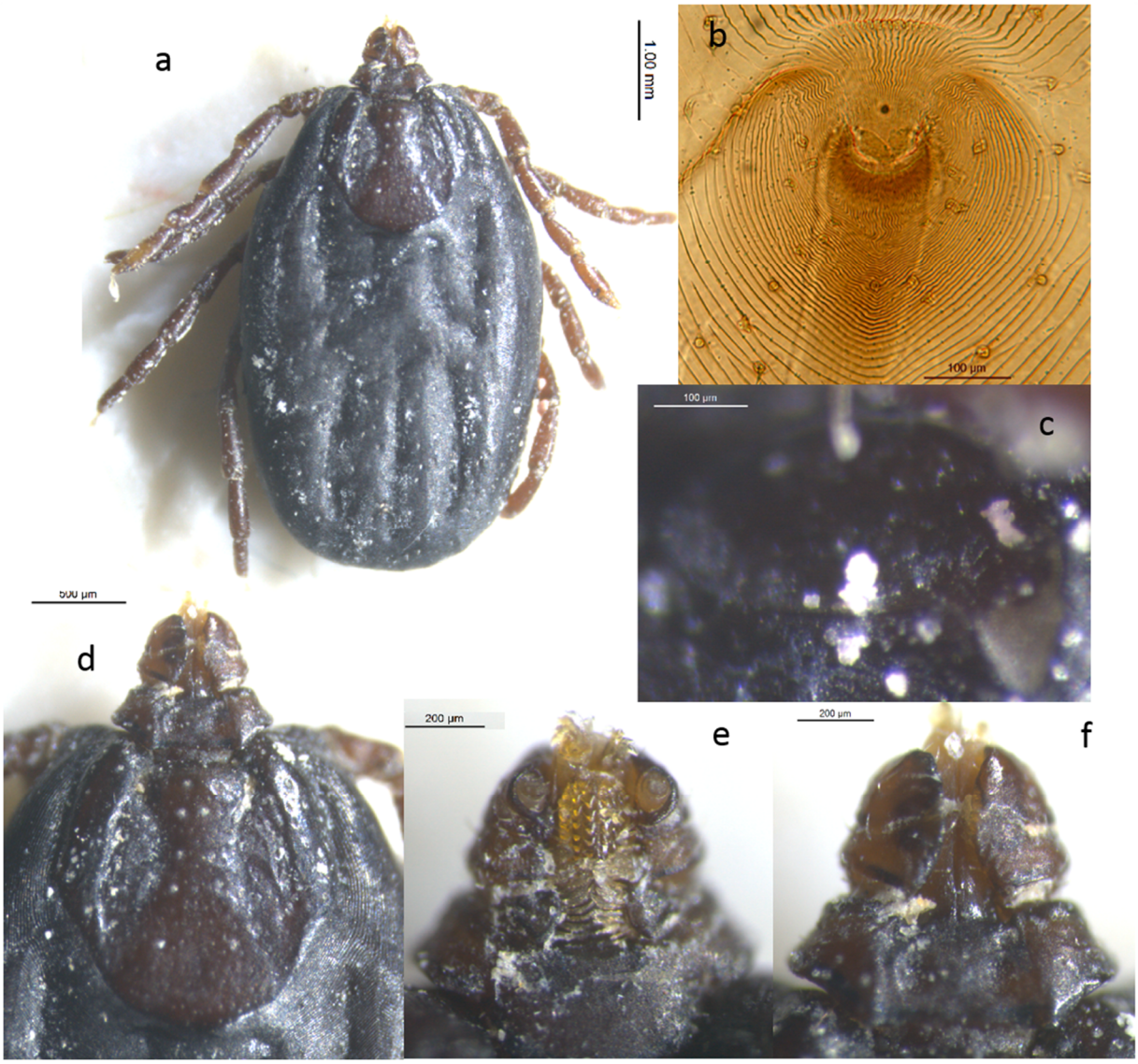

Fig.63 – Female tick, ID= CZ O108 (IICT). Classification according Dantas-Torres (2013): *R. sanguineus* African type. (a) Dorsal body view, evident engorgement; (b) genital aperture with a height U shape, with triangular, short and slightly sclerotized flaps, and short operculum; (c) spiracular plate, with spiracle tail pointed upward, and a tail height equal to half of the oval area height; (d) capitulum and a shorter scutum, with dense and small, as well as larger "*simus*" like punctations; (e) ventral view of capitulum; (f) dorsal view that evidence a shorter capitulum.

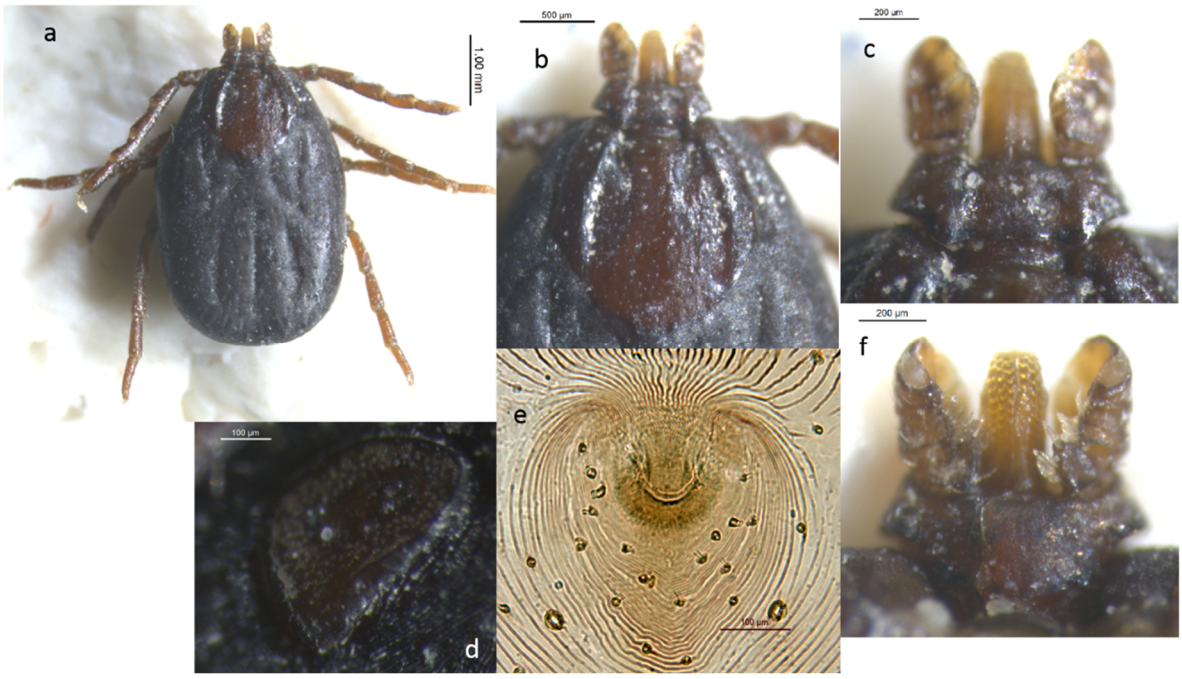

Fig.64 – Female tick, ID=CZ O109 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* type II. Observations:, (a) Dorsal body view, evidence a slightly engorgement; (b) capitulum and a shorter scutum, with dense and small, as well as a larger "*simus*" like punctations; (c) clearly short capitulum dorsal view; (d) spiracular plate, with spiracle tail pointed upward, and a tail height equal to half of the oval area height; (e) height U shaped genital aperture, with slightly sclerotize and long flaps, with a long operculum; (f) ventral view of capitulum.

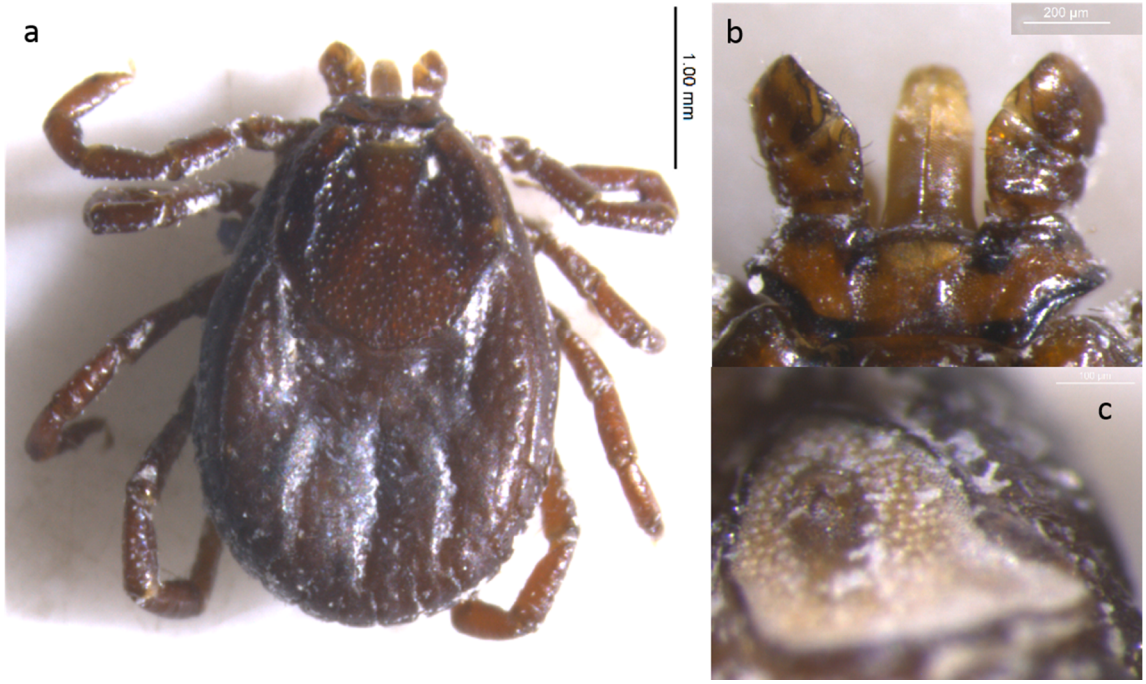

Fig.65 – Female tick, ID=CZ O141 (IICT). Evaluation according Travassos Dias (1994): *R. pusillus*. (a) body dorsal view, with a evident shorter scutum, with small and dense punctations, and a less evident "*simus*" like punctation; (b) small, higher than long capitulum, with the basis capituli evidence a large and continuous black outline; (c) spiracular plate, with spiracle tail pointed upward, and a tail height equal to half of the oval area height.

This observation can be explain based on many observations described in the literature that state the *R. sanguineus* seems to display a size variability in different regions (Inokuma et al., 1997; Jittapalapong et al., 2000; Oliveira et al., 2005; Ribeiro et al., 1996; Szabó et al., 2005). For example, a comparison of published data showed that engorged *R. sanguineus* females from Brazil may weigh 50% less than those from the North America and Japan (Inokuma et al., 1997; Jittapalapong et al., 2000; Szabó et al., 2005). Then, size and morphological variations among *R. sanguineus* are not an unexpected feature in such a widely distributed tick species. More specifically, morphological variation intra and interpopulations were detected in the adanal plates, genital aperture, spiracular plates, hypostome dentition and in palps of Brazilian *R. sanguineus* ticks (Ribeiro et al., 1996).

Focusing on the used variables, the ‘Spiracular angle’ was the best feature used in the females clusters distinction; the ‘Ventral-measured palp height’, ‘Basis capituli length/width ratio’, ‘Scutum length/width ratio’ and ‘Spiracular tail length/widths-difference ratio’ followed. Although not so useful, even the ‘Porose areas height/width ratio’ and the ‘Spiracle Oval area height/width ratio’, even so contributed to some degree of differentiation. The less valuable of all variables was the ‘Porose areas distance’ variable, which did not have any significance at differentiating the clusters. The only significant and with a strong effect on the female qualitative clusters formation was the “Scutum punctation size” variable. Despite some effect, the others turned to be weak variables to this purpose.

Using CA, it is possible to conclude that many female groups shared too many qualitative variables (exception made to “Scutum punctation size”). Despite that, it is still possible to say, using all the association results, that according to these results, the female *R. sanguineus* cluster formed with the traditional taxonomic identifications includes too many intraspecific variations to be clearly defined with this type of statistical analysis. Also, the intermediate female cluster and the female *R. turanicus* cluster formed in the same way have more in common than with *R. sanguineus* cluster.

- **Male Specimens Clusters**

During the formation of the males groups, was observed that the animal CZ S1236 formed an isolated group, what lead to its exclusion from the following analysis steps.

This separation may have occurred because this specimen exhibit features with different characteristics relatively to all the rest of the specimens, such as happen with the large values presented in the 'Post posteromedian groove width/conscutum width ratio' and 'Conscutum length/width ratio' variables. This means that this specimen have a longer but less wide conscutum than the other specimens, and this discrepancy is the reason of its isolation (see Fig.66). As already referred, morphological features size variations are normal in the *R. sanguineus* species, being often observed (Inokuma et al., 1997; Jittapalapong et al., 2000; Oliveira et al., 2005; Ribeiro et al., 1996; Szabó et al., 2005).

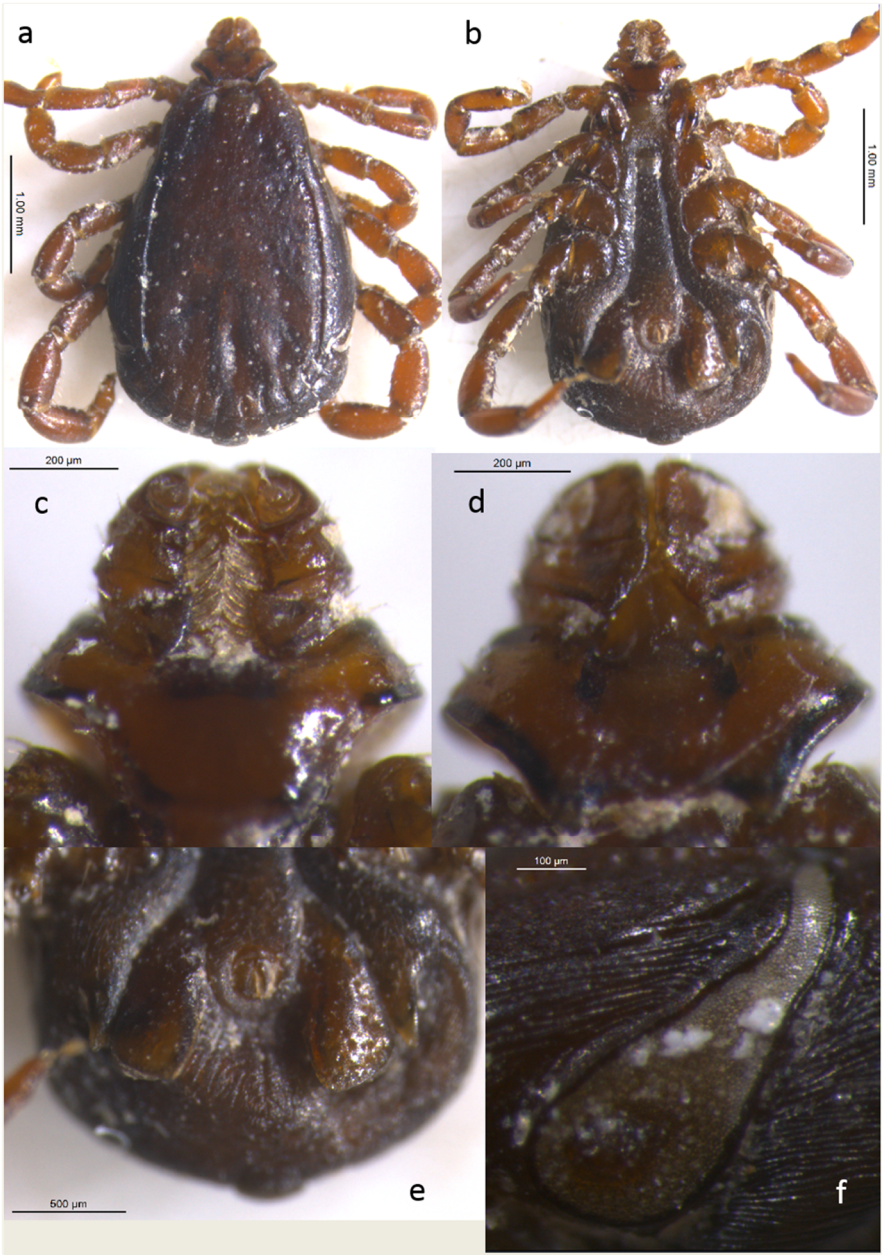

Fig.66 – Male tick, ID=CZ S1236 (IICT). Evaluation according Dantas-Torres (2013): African *R. sanguineus* type. (a) total body dorsal view, with a very discrepant long and narrow conscutum (length=3.606, width=1.122); (b) total body ventral view; (c) capitulum ventral view; (d) capitulum dorsal view, as long as wide; (e) average adanal plates; (f) spiracular plate, globular, with a long and thinner tail.

Regarding to the morphological variables, the 'Post posteromedian groove width/conscutum width ratio' was the best quantitative variable used in the males' clusters distinction; and the 'Adanal plates height/ width ratio' was the only one with no statistical significant contribution to this clustering. All the other features had a significant contribute to the clusters formation.

The adanal plates are normally considered as a differentiator feature (Dantas-Torres et al., 2013; Estrada-Peña et al., 2004; Ioffe-Uspensky et al., 1997; Pegram et al., 1987b; Walker et al., 2003), and this result is not in accordance with the results previously obtained. This may have happen due to the weak mathematical ratio used to describe quantitatively this morphological feature, so it is important to use more or different measures for obtain a best characterization.

The best qualitative variable was the 'Cervical field's depression', followed in descending order of contribution by the 'Parma presence', the 'Adanal plates posterior margin', the 'Cervical fields shape', the 'Cervical grooves definition', and the 'Adanal plates ending' variables. The 'Posteromedian grooves shape' was the less significant qualitative variable. The latter, adding to the 'Lateral grooves beginning', the 'Posteromedian grooves deepness', and the 'Posteromedian grooves length' that did not present statistical significance on the clusters formation, can be classified as poor variables for this clustering analysis, and are suggested to be put aside on a future analysis.

All the others qualitative variables were not found in a sufficient number of specimens what left no way to evaluate appropriately their contribution. Anyway, it is of note that the 'Adanal plates total shape' still had a high contribute, and is a variable to be considered in the analysis. Reevaluation of some less significant qualitative variables present in the present study may be another way to solve this issue.

Using CA it is still possible to say, after analysing all association results, that the male *R. sanguineus* cluster formed with the traditional taxonomic identifications has too many intraspecific variations to be clearly defined for this type of statistical analysis. Moreover, the intermediate male cluster is easily differentiated with the qualitative analysis, and the male *R. turanicus* cluster with the quantitative analysis.

5.2. Latest Morphological and Ultramorphological Analysis

Comparing the obtained male type-groups descriptions with the obtained results from the statistical analysis, is possible to said that: the specimens classified as *R. sanguineus* African type and as *R. sanguineus* type I are frequently founded in the same cluster (equivalent to the "*R. sanguineus*" cluster); the *R. sanguineus* type II and *R. sanguineus* type a. are frequently found on the *R. sanguineus* African type and *R. sanguineus* type I cluster, but also in another cluster (equivalent to the "Intermediate" cluster) due to some different morphological features presented, jointly with *R. turanicus* type b.; the *R. turanicus* and the *R. turanicus* type b. are frequently found in a third different cluster (equivalent to the "*R. turanicus*" cluster).

This shows a result in accordance with the stronger association referred before, in the statistical CA, between the "Intermediate" group (of the traditional taxonomic analysis) and the "*R. sanguineus*" group than with the "*R. turanicus*" group. That is, the highest concentration of *R. sanguineus* specimens (in particularly, *R. sanguineus* type II and *R. sanguineus* type a.) in the "Intermediate" group leads to a stronger association of this specific group with the "*R. sanguineus*" group in the CA (see Fig.36).

These results are consistent with some data given by Keirans, 2014 (via personal communication), saying that there could exist up to 8 species of *R. sanguineus s.l.* in the European continent, and possibly in Portugal.

Comparing the obtained female type-groups descriptions with the obtained results of the statistical analysis, is possible to say that: in the equivalent to the "*R. sanguineus*" cluster of the traditional taxonomic classification, were frequently founded the *R. sanguineus* African type, the *R. sanguineus* type II, A. and B.; in the equivalent to the "Intermediate" cluster, were also frequently founded the *R. sanguineus* African type, the *R. sanguineus* type II, and the *R. sanguineus* type C.; and in the equivalent to the "*R. turanicus*" cluster, were frequently founded the *R. sanguineus* type II, the *R. turanicus* A. and the *R. turanicus* type B.

These results evidence, as referred in the statistical analysis, the great morphological variability still present in the females' *R. sanguineus* group, grouping 4 morphological

differentiable type-specimens. Even so, is to note that the *R. turanicus* types are almost isolated from the *R. sanguineus* specimens, except from some *R. sanguineus* type II specimens (which is also the most common type-morphology on the studied specimens). The interesting issue here is that *R. sanguineus* type II is the most morphologically identical to the *Rhipicephalus* sp. II group described by Dantas-Torres et al. (2013), and considered as present in Portugal. However, more significant results need to be obtained to conclude more of this observation.

Moreover, the typical *R. turanicus* of the Mediterranean (ours *R. turanicus* type A., which are specimens that resembles Italian *R. turanicus* – data not shown) is less common in our study populations, which can indicate that in Portugal other morphologic variation of *R. turanicus* are more common (our study *R. turanicus*, which resembles more the African species).

Sum up, the obtained results in the carried out analyzes were more enlightening for males than for females, due to some reasons as: 1) the females were more outnumbered, because they are harder to find in a not engorged state; 2) females have less morphological descriptive features than males, which implies less enlightening results.

5.3. Molecular Analysis

The lack of success (efficiency of 11%) in obtaining appropriated genetic sequences in molecular analysis may be a result of the low specificity of COI primers used on the *R. sanguineus* and *R. turanicus* genetic sequences of Portuguese populations, especially due to the tremendous genetic variability expected (accordingly to the morphological variability observed) in this population.

The genetic analysis resulted in 21 specimens identified as *R. sanguineus* and 1 (ID =CZ CR1563) as *R. turanicus*. Accordingly with the traditional taxonomic analysis – based on conventional keys and descriptions of Travassos Dias (1994), Walker (2003) and Papadopoulos (1992) –, CZ CR1563 specimen it is not a *R. turanicus.* For more detail, see Fig.67 and the Fig.68, being the first the CZ CR1563 specimen and the last an African *R. sanguineus* specimen (female, Egypt, 1954) identified by H. Hoogstraal in 1957 and given to Tendeiro for the ZC of IICT, and by comparison is possible to say that these two specimen are very morphologic similar.

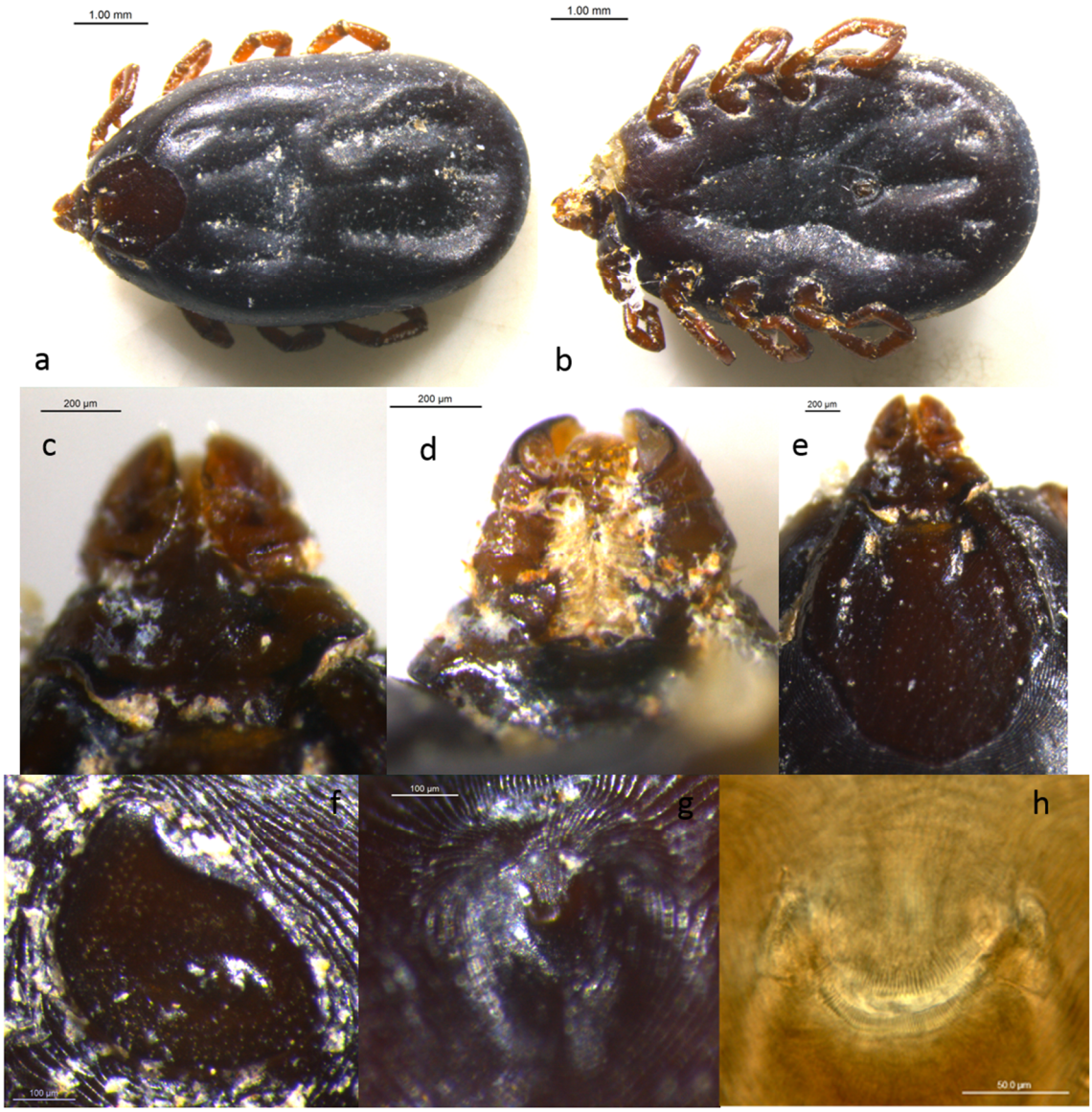

Fig.67 – Female tick, ID=CZ CR1563 (IICT). Evaluation according Dantas-Torres (2013): *R. sanguineus* African type. (a) body dorsal view; (b) body ventral view; (c) basis capituli and palps, dorsal view; (d) basis capituli, hypostome and palps, ventral view; (e) scutum and capitulum dorsal view; (f) spiracular plate, with a short tail; (g) genital aperture, open U shaped; (h) mounted genital aperture, with visible aparted triangular sclerites and open U shaped.

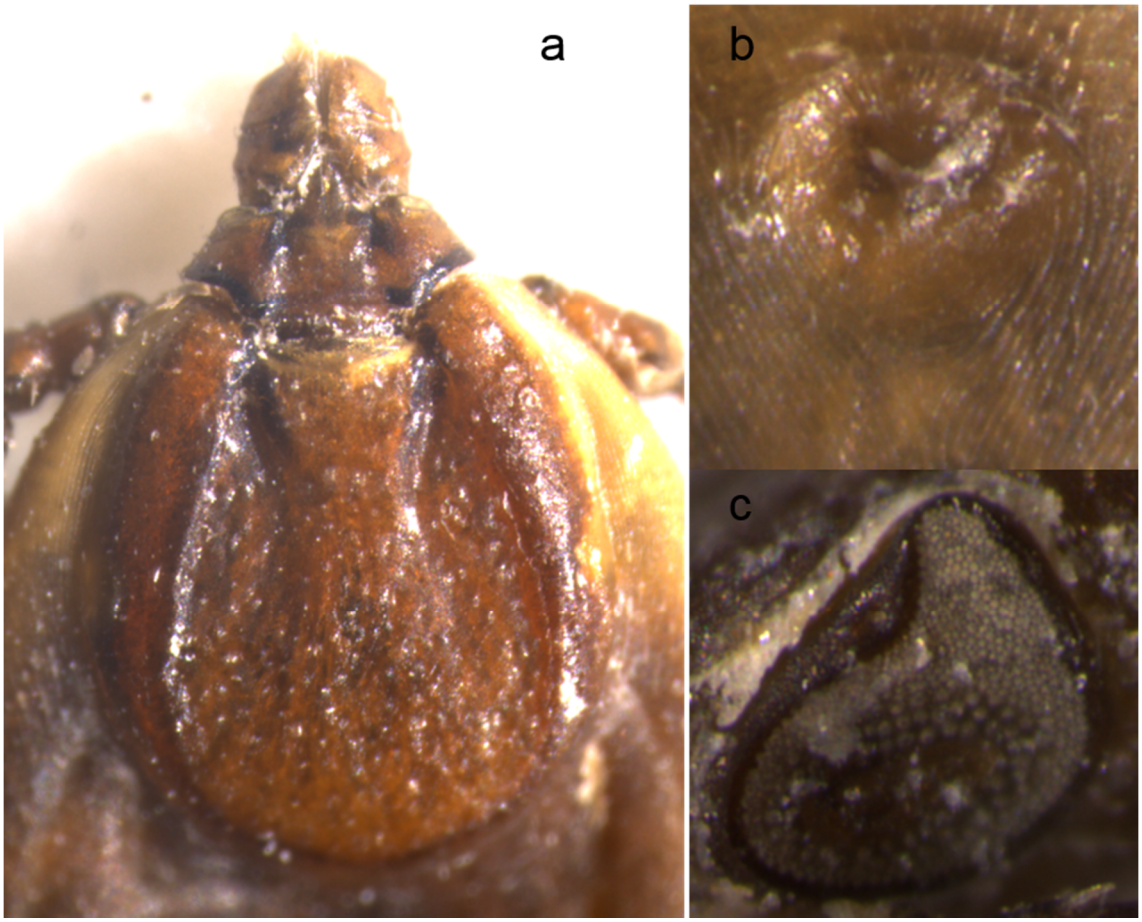

Fig.68 –African *R. sanguineus* specimen (female, Egypt, 1954) identified by H. Hoogstraal in 1957 and given to Tendeiro (ZC of IICT). Morphology identical to the CZ S1563 (the same collection).

This contradictory outcome may have been obtained due to several reasons: 1) the obtained homology percentage, the smallest of all those analyzed (91%), was not sufficient enough for a correct identification of the sequence; 2) the GenBank match genetic sequence, which is from a Chinese specimen, may not be an appropriated sequence for comparison with ours, either because it is from a completely different population and geographical niche (with different ecological, morphological and phylogenetic characteristics), or because it is not possible to access its morphology; 3) the sequences of GenBank are not revised by assessors, so there is no confirmation of the homologue sequence replicability; 4) sample also may not have the best quality to be assessed, and may contain some alterations or errors. Therefore, we are inclined to identify this specimen as *R. sanguineus* based on its morphological features.

For the same reasons, we think that at least the specimens S337 and CR1575 (IDs of ZC/IICT) resembles *R. turanicus*, although the results pointed the *R. sanguineus* as the species more probable.

Moreover, some contradictions on the literature (Rosa et al., 2013; Santos-Silva et al., 2011) are probably duo also due to misidentifications and limitations of mitochondrial genome dataset available in online databases, also referred by Dantas-Torres and collegues (2013).

One study on the multiple alignments of nearly 900 species of Ixodida conducted by Chinese researchers (Lv et al., 2014), with Chinese ticks populations, showed a high level of nucleotide variability in the COI sequences universal priming sites. Adding to this, it is also suggested that the primers pair LCO1490/HCO2198 could not be efficient in the amplification of the COI ticks sequence (Lv et al., 2014). These conclusions are in accordance with ours results.

Even so, there are currently several sets of primer pair for tick' COI sequence amplification that can be use in the future to get through the issue we faced. Some of those primers are shown in Table.11 (Lv et al., 2014). Other hypothesis is to use other sets of primers (such as 18S, 16S or ITS2), that could also be the answer to overcome this lack of specificity (see Table.11 for some examples).

Table.11 - COI, 16S rDNA, ITS2 and 12S rDNA primer pairs that can be used to Ixodida species identification, adapted from Lv et al (2014).

Gene	Primer Pairs	Forward	Reverse	Length of fragments	Reference
COI	COI-F/ COI-R	5'ATCATAAAKAY HTTGG 3'	5'GGGTGACCRAAR AAHCA 3'	Approximately 680 bp	Lv et al., 2014b
	TY-J-1449/ C1-N-2312	5'AATTTACAGTTT ATCGCCT3'	5'CATACAATAAAG CCTAATA3'	Approximately 860 bp	Rees et al, 2003
	Cox1F/ Cox1R	5'GGAACAATATAT TTAATTTTTGG3'	5'ATCTATCCCTACT GTAAATATATG3'	Approximately 820 bp	Chitimia et al, 2010
	HCO2064/ HCO1215	5'GGTGGGCTCATA CAATAAATCC3'	5'GCCATTTTACCGC GATGA3'	Approximately 860 bp	Song et al, 2011
16S	16S-F/ 16S-R1/	5'TTAAATTGCTGT RGTATT3'	5'CCGGTCTGAACTC ASAWC3'	Approximately 455 bp	Lv et al., 2014b
ITS2	ITS2-F/ ITS2-R	5'ACATTGCGGCCT TGGGTCTT3'	5'TCGCCTGATCTGA GGTCGAC3'	Ranged from 1200 to 1600 bp	Lv et al., 2014a
12S	T1B/T2A	5'AAACTAGGATTA GATACCCT3'	5'AATGAGAGCGAC GGGCGATGT3'	Approximately 320 bp	Beati and Keirans, 2001

Proceeding, theirs analysis also showed that genetic divergence in all of the four DNA markers used (COI, ITS2, 16S and 12S rDNA) between some congeneric species was very low (<0.05%) (Lv et al., 2014), what indicates that some species of the Chinese population cannot be distinguished among eachother based on this DNA fragments. For example, they could not distinguish *R. sanguineus* and *R. turanicus* from each other, while *R. microplus* and *R. annulatus* (which are proven to be two morphologically different species) proven to be difficult to be differentiated.

Recently, a study pointed out the existence of different species under the names *R. sanguineus* and *R. turanicus* (Dantas-Torres et al., 2013). According to the same study, the low resolving power of the DNA markers for the discrimination of these species might be caused by the very same taxonomic issue mentioned earlier. Therefore, it is important to consider the reclassification of the populations currently designated *"R. sanguineus"* and *"R. turanicus"*, like the one made by Dantas-Torres (2013), so that it can be determined by molecular means if these populations do or do not include different species.

6. CONCLUSION AND PERSPECTIVES

R. sanguineus is a complex of closely related species primarily based on traditional morphological characters identification, and still today, the status of this group systematics is unclear and is under intense debate, resulting in ticks' misidentification and in mistrust results. The great morphological variability documented between *R. sanguineus* and *R. turanicus*, within this complex, can be based on populations that present slightly different features alterations, especially for some non-fixated morphological traits (parma, form of palp II, adanal plates, spiracular plate), and that coexist in the same ecological habitat, raising the chances of crossing and hybridization.

The statistical analysis performed in this study is in agreement with many studies which said that the *R. sanguineus* have too much intraspecific variability to be considered just only one species, and even that the specimens under the *R. turanicus* name are, in fact, another morphological group to take into account. Also in ours *R. turanicus* specimens we obtain the indication that in Portugal the most common morphologic variation group resembles more the African that the Mediterranean *R. turanicus*, which have to be studied further.

For future work proposals, it is suggested to not include in the statistical study the less significant variables used here, and it is proposed to reclassify the qualitative variables, to see if that lead us to achieve different or more significant results. It is also suggested that, for obtain more accurate outcome from the females analysis, more morphological descriptive variables need be applied in this genus (as the measure of sclerites, the genital aperture height and width), and to increase the studied species number, to may also obtain a more positive outcome.

Primer pair COI (LCO1490/HCO2198) used in this study was not efficient in the recovery of sequences from tick specimens, and it is important in the future to analyze more *Rhipicephalus spp.* individuals to see if the issue still persists. Phylogenetic studies are also a good bet to surpass this misidentification issue in a genetic context.

This study allowed us then to prove that statistic analysis, by obtaining reproducible results, can be a big auxiliary when it comes to evaluating associations between highly

morphological variable groups, and even if there is a great variation within a species that worth to explore.

R. sanguineus is undoubtedly one of the most important species from the veterinary and medicine standpoints in Portugal. Since different *Rhipicephalus* spp. may differ in their ability to transmit PA to both animals and humans, accurate species identifications are crucial, since they represent the basis for the establishment of effective programs to monitor tick populations, as well as to develop sustainable treatment and control strategies against TBD.

But still today some aspects of these ticks taxonomy and biology linked to molecular systematics and genetics, as many other aspects, are still missing or poorly covered. So, the development of works like this are essential and important in the upgrade of knowledge in this study area.

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APPENDICES

APPENDIX I – MOLECULAR PROTOCOL

Gene: Cytochrome oxidase I (COI)

Length: Approximately 700 bp

Primers: 25 nmole DNA Oligo

LCO-1490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'
HCO-2198	5-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'

PCR protocol:

1. Create master mix containing:

9.90 µl	Water
5.00 µl	5X buffer
1.00 µl	dNTPs (2.5mM)
1.00 µl	Primer LCO-1490 (10 µM)
1.00 µl	Primer HCO-2198 (10 µM)
2.00 µl	BSA (2 µM)
2.00 µl	$MgCl_2$ (25mM)
0.10 µl	Taq

2. Add 22 µl of master mix to each PCR tube
3. Add 3 µl DNA template for total of 25 µl per PCR reaction

PCR program:

Duration – approximately 3 hours

1. 94°C / 1 min.
2. 5 cycles of:
    a. 94°C / 30 sec.
    b. 45°C / 1 min.
    c. 72°C / 1 min.
3. 35 cycles of:
    a. 94°C / 1 min.
    b. 50°C / 1 min. 30 sec.
    c. 72°C / 5 min.
4. 72°C / 5 min.
5. Hold at 4°C

Run PCR product on normal gel (4 µl DNA + 2 µl dye) and note band intensity.

Clean successful amplifications using SureClean Plus Kit (Bioline Company).

Samples ready for sequencing are send to Macrogen Europe.

APPENDIX II – COEFFICIENTS HCA TABLE

Table I. 12 – The last 10 fusion coefficients obtained in the Hierarchical Cluster Analysis. Used to choose the number of clusters to obtain in the different statstical analysis effectuated. N- number, V. - variable.

N Clusters to Form	Fusion Coeficients			
	Females		Males	
	Quantitative V.	Qualitative V.	Quantitative V.	Qualitative V.
2	1088,000	54,206	2592,000	264,946
3	958,751	46,515	2366,676	235,129
4	857,868	40,810	2153,106	217,505
5	782,057	35,109	1968,592	206,479
6	718,303	31,511	1852,717	201,080
7	664,245	27,927	1748,074	196,590
8	627,167	24,876	1665,216	192,890
9	591,411	22,779	1584,445	189,246
10	560,693	21,117	1521,246	185,917
11	532,631	19,704	1463,038	182,636

APPENDIX III – STATISTIC DESCRIPTIONS

Section I:

To classify the formed females' qualitative variables clusters, from the 137 studied specimens of this gender, a cross-tabulation statistics was performed. The females' qualitative variables clusters characterization by percentages is describe for each cluster down:

Cluster 1 – Of the 40 elements (29.2% of the 137 female specimens) within this cluster: 29 (72.5%) have second palps square-shaped, 11 (27.5%) have second palps more long in width than in height; 35 (87.5%) have dense scutum punctation distribution, 4 (10.0%) have sparse scutum punctation distribution, 1 (2.5%) present localized scutum punctation distribution; 34 (85.0%) have small sized scutum punctation, 6 (15.0%) have small and medium sized scutum punctations; 1 (2.5%) have smooth scutum posterior margin shape, 26 (65.0%) present slightly sinuous scutum posterior margin shape, 13 (32.5%) have sinuous scutum posterior margin shape; 2 (5.0%) present small cervical fields shape (the only group with this feature), 28 (70.0%) have large and curved cervical fields shape, 10 (25.0%) have large and straight cervical fields shape; 22 (55.0%) present small setiferous punctations, 15 (37.5%) present large setiferous punctations, 3 (7.5%) present small and large setiferous punctations; 1 (2.5%) do not present cervical grooves and 39 (97.5%) have defined cervical grooves.

Cluster 2 – Of the 36 elements (26.3% of the total) within this cluster: 6 (16.7%) have second palps square-shaped, 30 (83.3%) have second palps more long in width than in height; 17 (47.2%) have smooth scutum posterior margin shape, 19 (52.8%) present slightly sinuous scutum posterior margin shape; 9 (25.0%) have a large and curved cervical fields shape, 27 (75.0%) present a large and straight cervical fields shape; 33 (91.7%) have small setiferous punctations, 3 (8.3%) have large setiferous punctations; 1 (2.8%) present mild cervical grooves, and 35 (97.2%) present defined cervical grooves. All group members present a dense and small sized scutum punctation.

Cluster 3 – Of the 19 elements (13.9% of the total) within this cluster: 10 (52.6%) have second palps square-shaped, 8 (42.1%) have second palps more long in width than in height, 1 (5.3%) have second palps more long in height than in width; 17 (89.5%) present

a dense scutum punctation distribution, 2 (10.5%) present a sparse scutum punctation distribution; 3 (15.8%) have a small and medium sized scutum punctations; 16 (84.2%) have small, medium, and large sized scutum punctations; 4 (21.1%) have smooth scutum posterior margin shape, 10 (52.6%) have a slightly sinuous scutum posterior margin shape, 5 (26.3%) present a sinuous scutum posterior margin shape; 12 (63.2%) have a large and curved cervical fields shape, and 7 (36.8%) of them present a large and straight cervical fields shape. All group members present small setiferous punctations and defined cervical grooves.

Cluster 4 – Of the 42 elements (30.7% of the total) within this cluster: 21 (50.0%) have slightly sinuous scutum posterior margin shape, 21 (50.0%) present a sinuous scutum posterior margin shape; 31 (73.8%) have a large and curved cervical fields shape, and 11 (26.2%) have a large and straight cervical fields shape. All group members present dense distribution and small sized scutum punctations (setiferous and no-setiferous), defined cervical grooves, second palps more long in width than in height.

Section II:

To classify the formed males' qualitative variables clusters, from the 288 studied specimens of this gender, a cross-tabulation statistics was performed. The males' qualitative variables clusters characterization by percentage it is describe for each cluster below:

Cluster 1 – Of the 76 elements (26.4% of the 288 male specimens) within this cluster**:** 74 (97.4%) present dense conscutum punctation distribution, 2(2.6%) present sparse punctation distribution; 43 (56.6%) present small conscutum sized punctations, 31 (40.8%) have small and medium sized punctations, 2 (2.6%) have small, medium and large sized punctations; All members present cervical fields depressions; 1 (1.3%) do not present a cervical fields shape, 31 (40.8%) present a small cervical fields shape, 23 (30.3%) present a large and curved cervical fields shape, 21 (27.6%) present a large and straight cervical fields shape; 1 (1.3%) do not present setiferous punctations, 35 (46.1%) present small setiferous punctations, 37 (48.7%) present large setiferous punctations, 3(3.9%) present small and large setiferous punctations; 29 (38.2%) present mild cervical grooves, 47(61.8%) present defined cervical grooves; 4 (5.3%) have the second palp square-shaped, 30 (39.5%) have the second palp long in width, 42 (55.3%) have the second palp long in height; 18 (23.7%) present lateral grooves beginning immediately after the eye, 58

(76.3%) present lateral grooves beginning distant of the eye; 20 (26.3 %) have the lateral grooves ending in the 1st festoon, 56 (73.7 %) have the lateral grooves ending in the 2nd festoon; 3 (3,9%) present lateral grooves with smooth-texture, 60 (78,9%) present lateral grooves with punctate-texture, 13 (17,1%) present lateral grooves with distinctly punctate-texture; 21 (27,6%) present short posteromedian grooves, 55 (72,4%) present long posteromedian grooves; 12 (15,8%) present shallow posteromedian grooves, 64 (84,2%) present deep posteromedian grooves; 24 (31,6%) present shallow paramedian grooves, 52 (68,4%) present deep paramedian grooves; 11 (14,5%) present circular-shaped paramedian grooves, 25 (32,9%) present oval-shaped paramedian grooves, 30 (39,5%) present comma-shaped paramedian grooves, 10 (13,2%) present long-shaped paramedian grooves; All members present parma; 59 (77,6%) present adanal plates posterior margin square-shaped, 17 (22,4%) present adanal plates posterior margin round-shaped, 59 (77,6%) present adanal plates total form square-shaped, 5 (6,6%) present adanal plates total form round-shaped, 12 (15,8%) present adanal plates total form with intermediate form between round and square-shape; 35 (46,1%) present a short adanal plates end, 41 (53,9%) present a long adanal plates end.

Cluster 2 - Of the 51 elements (17.7% of the total specimens) within this cluster**:** 49.9 (96.1%) present dense punctation distribution, 2 (3.9%) present sparse punctation distribution; 28 (54.9%) present small conscutum sized punctations, 22 (43.1%) have small and medium sized punctations, 1 (2.0%) have small, medium and large sized punctations; All members do not present cervical fields depressions; 49 (96.1%) do not present cervical fields shape, 2 (3.9%) present a small cervical fields shape; 1(2%) do not present setiferous punctations, 24(47.1%) present small setiferous punctations, 25(49%) present large setiferous punctations, 1(2%) present small and large setiferous punctations; 47(92.2%) present mild cervical grooves, 4(7.8%) present defined cervical grooves; 1(2.0%) have the second palp square-shaped, 17(33.3%) have the second palp long in width, 33(64.7%) have the second palp long in height; 14 (27.5%) present lateral grooves beginning immediately after the eye, 37 (72.5%) present lateral grooves beginning distant of the eye; 15 (29.4%) have the lateral grooves ending in the 1st festoon, 35 (68.6 %) have the lateral grooves ending in the 2nd festoon, 1 (2.0 %) have the lateral grooves ending before the 1st festoon; 47 (92,2%) present lateral grooves with punctate-texture, 4 (7,8%) present lateral grooves with distinctly punctate-texture; 15 (29,4%) present short posteromedian grooves, 36 (70,6%) present long posteromedian grooves; 13 (25,5%) present shallow posteromedian grooves, 38 (74,5%) present deep posteromedian grooves; 19 (37,3%) present shallow paramedian grooves, 32 (62,7%) present deep paramedian grooves; 12 (23,5%) present circular-shaped paramedian grooves, 17 (33,3%) present oval-shaped paramedian grooves, 21 (41,2%) present comma-shaped paramedian grooves, 1 (2,0%) present long-shaped paramedian grooves; 31 (60,8%) do not present parma, 20 (39,2%) present parma; 41 (80,4%)present adanal plates posterior margin

square-shaped, 10 (19,6%) present adanal plates posterior margin round-shaped, 38 (74,5%) present adanal plates total form square-shaped, 13 (25,5%) present adanal plates total form with intermediate form between round and square-shape; 24 (47,1%) present a short adanal plates end, 27 (52,9%) present a long adanal plates end.

Cluster 3 - Of the 123 elements (42.7% of the total specimens) within this cluster**:** 121 (98.4%) have dense punctation distribution, 2 (1,6%) have localized punctation distribution; 74 (60.2%) present small conscutum sized punctations, 49 (39.8%) have small and medium sized punctations; 9 (7.3%) do not present cervical fields depressions, 114 (92.7%) present cervical fields depressions; 26 (21.1%) present a small cervical fields shape, 40 (32.5%) present a large and curved cervical fields shape, 57 (46.3%) present a large and straight cervical fields shape; 67(54.5%) present small setiferous punctations, 54(43.9%) present large setiferous punctations, 2(1.6%) present small and large setiferous punctations; 51(41.5%) present mild cervical grooves, 72(58.5%) present defined cervical grooves; 1(0.8%) have the second palp square-shaped, 22(17.9%) have the second palp long in width, 100(81.3%) have the second palp long in height; 39(31.7%) have small and medium sized punctations; 84 (68.3%) present lateral grooves beginning distant of the eye; 29 (23.6%) have the lateral grooves ending in the 1st festoon, 94 (76.4%) have the lateral grooves ending in the 2nd festoon; 2 (1,6%) present lateral grooves with smooth-texture, 110 (89,4%) present lateral grooves with punctate-texture, 11 (8,9%) present lateral grooves with distinctly punctate-texture; 41 (33,3%) present short posteromedian grooves, 82 (66,7%) present long posteromedian grooves;19 (15,4%) present shallow posteromedian grooves, 104 (84,6%) present deep posteromedian grooves; 29 (23,6%) present shallow paramedian grooves, 94 (76,4%) present deep paramedian grooves; 10 (8,1%) present circular-shaped paramedian grooves, 47 (38,2%) present oval-shaped paramedian grooves, 59 (48,0%) present comma-shaped paramedian grooves, 7 (5,7%) present long-shaped paramedian grooves; 2 (1,6%) do not present parma, 121 (98,4%) present parma; 116 (94,3%) present adanal plates posterior margin square-shaped, 7 (5,7%) present adanal plates posterior margin round-shaped, 119 (96,7%) present adanal plates total form square-shaped, 2 (1,6%) present adanal plates total form round-shaped, 2 (1,6%) present adanal plates total form with intermediate form between round and square-shape; 105 (85,4%) present a short adanal plates end, 18 (14,6%) present a long adanal plates end.

Cluster 4 - Of the 38 elements (13.2% of the total specimens) within this cluster**:** All elements have dense punctation distribution. 15(39.5%) present small conscutum sized punctations, 23(60.5%) have small and medium sized punctations; All members present cervical fields depressions; 10(26.3%) present a small cervical fields shape, 17(44.7%) present a large and curved cervical fields shape, 11(28.9%) present a large and straight cervical fields shape;

11(28.9%) present small setiferous punctations, 27(71.1%) present large setiferous punctations; 10(26.3%) present mild cervical grooves, 28(73.7%) present defined cervical grooves; 2(5.3%) have the second palp square-shaped, 8(21.1%) have the second palp long in width, 28(73.7%) have the second palp long in height;18 (47.4%) have small and medium sized punctations; 20 (52.6%) present lateral grooves beginning distant of the eye; 7 (18.4%) have the lateral grooves ending in the 1st festoon, 31 (81.6%) have the lateral grooves ending in the 2nd festoon; 30 (78,9%) present lateral grooves with punctate-texture, 8 (21,1%) present lateral grooves with distinctly punctate-texture; 14 (36,8%) present short posteromedian grooves, 24 (63,2%) present long posteromedian grooves; 7 (18,4%) present shallow posteromedian grooves, 31 (81,6%) present deep posteromedian grooves; 6 (15,8%) present shallow paramedian grooves, 32 (84,2%) present deep paramedian grooves; 6 (15,8%) present circular-shaped paramedian grooves, 16 (42,1%) present oval-shaped paramedian grooves, 16 (42,1%) present comma-shaped paramedian grooves; All members present parma; All members have adanal plates posterior margin rounded; 6 (15,8%) present adanal plates total form round-shaped, 32 (84,2%) present adanal plates total form with intermediate form between round and square-shape; 33 (86,8%) present a short adanal plates end, 5 (13,2%) present a long adanal plates end.